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The effect of oscillatory mechanical stimulation on osteoblast attachment and proliferation

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Abstract

The aim of this paper is to investigate the effect of magnitude and duration of oscillatory mechanical stimulation on osteoblast attachment and proliferation as well as the time gap between seeding and applying the stimulation. Cells were exposed to three levels of speed at two different conditions. For the first group, mechanical shear stress was applied after 20 minutes of cell seeding. For the second group there was no time gap between cell seeding and applying mechanical stimulation. The total area subjected to shear stress was divided into three parts and for each part a comparative study was conducted at defined time points. Our results showed that both shear stress magnitude and time gap between cell seeding and shear stress applying, are important in further cell proliferation and attachment. The effect of shear stress was not significant at lower speeds for both groups at the earlier time points. However, a higher percentage of area was covered by cells at the later time points under shear stress. In addition, time gap can also improve osteoblast attachment. For the best rate of cell attachment and proliferation, the magnitude of shear stress and time gap should be optimized. The results of this paper can be utilized to improve cell attachment and proliferation in bioreactors.

Keywords

Mechanical stimulation; osteoblasts; attachment; proliferation; orbital shaker; shear stress

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Conflict of interest statement

There are no conflicts of interest.

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1. Introduction

Shear stress is commonly considered a critical factor affecting specific cell lines such as endothelial cells and osteoblasts. Morphology and function of cells can be changed by shear forces [1–4]. Cells elongate and orient with uniaxial steady flow direction, while unsheared cells have a randomly oriented cobblestone appearance. Gradually increased steady shear stress reduces cell detachment. A very wide span of cellular phenomena is recognized as being influenced by fluid shear, including both mechanoreception (e.g., plasma membrane receptors, ion channels, integrins/focal adhesions, protein kinase signaling) and response (e.g., intracellular calcium, nitric oxide, prostacyclin, cytoskeletal remodeling) [5,6]. It was reported that axial spreading of cells is increased by shear stress and mechanical stimulation, but transverse spreading is unaffected [7,8]. Previous studies [9,10] have shown that cells in steady shear stress constantly rearrange their positions with no net migration. However, cells subjected to large spatial gradients of shear stress behaved for cell proliferation, shape changes, and actively migration away from offending locations. Randomness of the repetitive shear stress in turbulent flow may also be a factor. However, spatial fluctuations have been shown to inhibit elongation and alignment.

Due to the complexity of the *in vivo* studies, cellular response to a wide range of phenomena including mechanical stimulation has initially studied with *in vitro* conditions [11]. Laboratory apparatuses have been customized for applying specific mechanical stimulation such as hydrostatic pressure, fluid shear stress and substrate strain. One of the common strategies in bone tissue engineering is the *in vitro* expansion of cells on a scaffold before implantation into the body. Bioreactor systems have been widely used to improve osteoblast attachment for bone tissue engineering. Applying shear stress during cell attachment and proliferation can be considered as a method of accelerating and enhancing osteoblastic differentiation. Magnitude and pattern of shear stress (e.g. oscillatory and continuous flow) are shown to induce significant effect on cell behavior [12,13]. Nearly all long-term investigations analyzed a continuous flow while most of the short-term studies utilize pulsatile or oscillatory flow regime. A number of experimental setups have been used to study the effect of shear stress on cells with different types of shear forces [14–17]. Parallel plate flow chambers generate uniaxial shear simply and controllably. However, care must be taken into account for entrance length and the variation of shear across the transverse direction in the channel. The cone and plate apparatus give nearly uniform tangential shear field when operated at moderate to low speeds. Oscillatory or pulsatile flow can be created in either cone or plate apparatus with special pumps and drive motors. A disadvantage of both is that only one experiment can be performed at a time, which may cause long time requirements for large arrays of experiments. On the other hand, orbital shakers can be used to investigate many cases simultaneously. In addition, because of the pulsatile nature of blood flow in body, oscillatory flow mimics the mechanical shear experienced by bone cells. Due to the rotation of the plates in orbital shakers, it induces the shear force to the fluid. Fluids cannot withstand shear forces and the fluid inside the plate moves and produces oscillatory shear stress. The magnitude of shear stress is theoretically maximum at the outer region of the plate and minimum at the center [18].

Although some scientists have researched the effect of shear stress on osteoblasts [11], answers of some principle questions are still vague. For example, what is the optimum shear stress to reach the maximum cell attachment? What is the optimum time gap between cell seeding and applying shear stress to reach the maximum cell attachment?

To the best of author's knowledge, there is a lack of comprehensive study utilizing cyclic shear stress on osteoblasts. In addition, the effect of time gap between cell seeding and applying shear stress has not been fully discovered. In this study, effects of oscillatory shear stress produced by orbital shaker on murine osteoblast and time gap between cell seeding and applying shear stress are presented. It is hypothesized that both shear stress magnitude and time gap between cell seeding and applying the shear stress have significant effect on cell proliferation and attachment.

2. Materials and methods

2.1. Cell culture and preparation

Murine osteoblast (OB-6 line) vials were received from Dr. Lecka Czernik, at The University of Toledo. In order to prepare cell culture medium, alpha minimum essential medium (α -MEM) supplemented with 9% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen Strep) were purchased from Gibco. Each vial of osteoblasts was plated on Petri dishes with 100 mm diameters and incubated at 37°C in a humidified 5% CO₂/95% air atmosphere in an osteogenic medium. Cells were monitored and the medium was changed every 2–3 days. When the dishes reached 80% confluence, the adherent osteoblasts were harvested as follows: cells were washed twice with Hank's balanced salt solution, treated with 2 consecutive applications of trypsin/EDTA for 3–5 min. each at room temperature and washed with the growth medium. The total cell number of 20,000 was added to the well of the 24 well cell culture plate with flat bottom.

2.2. Shear stress applications

Different magnitudes of rotational speed (i.e., 50, 100, 150 and 200 rpm) was applied by using an orbital shaker (Innova 2000; New Brunswick scientific, Edison, NJ). The orbital shaker was carefully sterilized and secured inside the incubator.

To investigate the effect of time gap between cell seeding and applying shear stress, two main groups were examined. In the first group, shear stress was applied right after cell seeding and the other group cells were subjected to the mechanical stimulation after waiting 20 min to attach cells onto the plate. Multiple time gaps were tried (i.e. 2, 6, 12 and 24 h) and osteoblast behavior was not significantly different in terms of the area covered by cells in the following examined time points (data not shown). For each group, three samples (n=3) were examined. In addition, for each sample, images were taken in three different directions along the well diameter. For each of the mentioned group, the experiment was carried out without applying shear stress (control) and 6, 24 and 48 h of shear stress exposure. In other words, the effects of oscillatory mechanical stimulation and time gap before applying the shear stress exposed on bone cells were investigated at three time points.

Orbital shakers can be used to investigate the effect of oscillatory shear stress on bottom of the orbiting dish has been previously described [19]:

$$\tau = a \sqrt{\rho \cdot \mu \cdot (2\pi f)^3}$$

Where τ is the maximum shear stress at the bottom of the dish, a is the radius of orbit, ρ is the fluid density, μ is the fluid viscosity, and f is the frequency of rotation (rounds/sec).

We assumed cell culture medium has fluid properties similar to water. Therefore, viscosity and density are respectively, 8.94×10^{-4} Pa.s and 1000 kg/m^3 . The radius of orbit was measured to be 19 mm. Although some other parameters like dish geometry, effects of gravity and fluid volume have not been incorporated in the equation. A comparison with the model solved using computational fluid dynamics method showed an acceptable accuracy for small dimensional circular dishes [20].

2.3. Viability assay, microscopy and image analysis

Live/dead cell assay was utilized to identify spread viable cells in the total area of the well plate. By using this assay, cells were characterized by immunofluorescent staining of live cells (Calcein-AM, Invitrogen) in green and dead cells (Ethidium homodimer, Invitrogen) in red. Cell images were captured by Olympus FSX-100 fluorescence microscope at 4X magnification. The areas covered by cells for each well plate were measured by using ImageJ (National Institute of Health, Bethesda, MD) software. To compare among different groups, two-way analysis of variance (ANOVA) was performed by use of SPSS 17 and $p < 0.05$ was considered as statistically significant.

For the accurate measurement purpose, each well was divided into three areas (Fig. 1); the most outer part of the well area defined as a 2 mm thick strip (Edge). The middle part was a 4 mm thick strip (Periphery) and the inner circle with 2 mm radius. During the preliminary study, different cell behavior was observed within these three regions due to the magnitude of exposed shear stress. In each direction, eight images were captured along the well plate diameter and the procedure was repeated three times for each well in distinct directions.

3. Results and discussion

In this section, cell behavior when exposed to different magnitudes of shear stresses will be discussed.

At 50 rpm

At speed of 50 rpm, the magnitude of maximum shear stress was small. The calculated value of shear stress at 50 rpm speed was 2.25 dynes/cm^2 . Based on the captured images, cells aggregated at the center and edge of the wells when samples exposed to shear stress after 20 min time gap. This is attributed to the magnitude and fluctuation of the shear stress. Theoretically, the shear stress near the well plate wall is zero and higher percentage of the area covered by cells at different time points (Fig. 2A). The number of attached cells was small at the periphery region where the shear stress was maximum and the highest

fluctuation of shear stress was observed (Fig. 2B). In addition, center region had the highest number of attached cells due to the low shear stress and fluctuation (Fig. 2C). In fact, applying shear stress caused the cells to migrate from higher shear stress regions to the lower shear stress regions.

Although this phenomenon was observed for all time points, the effect of oscillatory shear stress was not significant ($p>0.05$) in the total cell area, which is a scale of the total number of attached and spread cells (Fig. 3A). Higher fraction of well area was covered by cells at center and edge regions showing the migration of cells due to the shear force (Fig. 3B). Comparatively, small percentage of total area was covered by cells at the periphery region.

The same pattern for cell attachment was observed for all the selected time points. For example, after 6 h of shear stress exposure, the average total area covered by cells was 3.8%, 6.3% and 5.5% of the total captured image for periphery, center and edge regions while the average area of the images from various well regions was 5.3% for the control samples. No significant difference in cell attachment was observed after 6 h ($p>0.05$). However, at higher time points, the difference in the area covered by cells was more significant ($p<0.05$). Although overall results showed the magnitude of exposed shear stress was small, the majority of cells did not extend extracellular matrix and vinculin protein to attach to the well plate substrate. Instead, cells migrated to the areas with the lowest shear stress. In the areas with lowest shear stress (center and edge), fluid kinetic energy was not enough to move and relocate cells, and the cells settled down in those areas.

Samples without time gap did not demonstrate big difference from those with time gap (Fig. 3A and Fig. 3B). The total number of cells was similar to the control sample in all measured time points. In addition, visual cell observation showed that cell behavior similar to the samples with 20 min time gap (Fig. 2). Therefore, those images were not shown here. Considering the covered area by cells in different regions in the well, slightly more cells migrated to the center of the well in comparison to the samples with the 20 min time gap.

100 rpm

The theoretical value for shear stress estimated at 100 rpm rotational speed was 6.45 dynes/cm². The results of applied shear stress on cells are shown in Fig. 4. The captured images for both groups (with 20 min time gap and without time gap) are available in supplementary data section (Figs. S1 and S2). In this speed, significant difference in the average of total cell area was observed between the control and other groups after 48 h ($p<0.05$). The average percentage of area covered by cells in the presence of shear stress exceeds almost 37% while in control samples the average percentage of area was about 25% (Fig. 4A). At other time points, although the percentage of area covered by cells was increased, the statistical analysis did not show a significant difference ($p>0.05$). In addition, after applying shear stress for 24 h, significantly higher number of cells was attached to the center and edge regions of the well for both samples with and without 20 min time gap ($p<0.05$) (Fig. 4B and Fig. 4D). Similar to the previous observations, significant differences were seen between edge, periphery and center regions. Cells migrated to the center due to the shear force stimulation ($p<0.05$). In addition, applying shear stress increased the total area covered by cells.

At this speed the total percentage of area covered by cells was increased. At 48 h, almost 40% of the total well plate area was covered by attached cells for the sample with 20 min time gap. This value was about 36% for the samples without time gap. This shows that within 20 min, cells started attaching to the surface and developing vinculin protein for cell attachment. In addition, this time gap helped more cells to attach.

150 rpm

The magnitude of applied shear stress for 150 rpm speed was 11.82 dynes/cm². At this rotational speed, the effect of mechanical stimulation was appeared at the earlier time points. As shown in Figure 5, significantly higher percentage of well area was covered by cells after 24 h in comparison with the control samples ($p < 0.05$), when exposed to the shear stress with 20 min time gap. The microscopic images are available at the supplementary data section (Fig. S3). On the other hand, applying shear stress without time gap does not provide sufficient time for cells to develop adhesion proteins. Therefore, total number of attached cells is lower in comparison with the samples exposed to shear stress after 20 min time gap. The corresponding microscopic images are shown in Fig. S4. Interesting phenomenon was observed for sample exposed to shear stress without time gap. Cells started aggregating to each other and made bigger spheres with the approximate diameter of few hundred micrometers. This phenomenon can be contributed to the higher magnitude of applied shear stress. At this speed cells attached together because they did not settle down on the well plate surface due to the high magnitude of shear stress. Upon aggregating and forming bigger cell colonies, they were heavy enough to overcome the force applied by shear stress and settled down and attached to the surface. This observation was not observed for samples exposed to shear stress after 20 min time gap, showing osteoblasts developed extracellular matrix components and attaching proteins from the early time of seeding. Extracellular matrix was shown to be an essential cell attachment component [21]. During cell attachment, cell produces specific proteins such as vinculin to develop extracellular matrix and subsequently attach to the substrate.

200 rpm

The magnitude of shear stress for 200 rpm rotational speed was calculated about 18.2 dynes/cm². Figure 6 shows the results for 200 rotational speed with 20 min time gap. We could not detect any cells attached to the well plate for the samples exposed to 200 rpm without time gap. This is because of the high shear stress induced to the osteoblasts. In this speed, cells could not attach to the well plate even after 48 h. The magnitude of shear force was too high to allow cells to attach and develop extracellular matrix components. In addition, the samples with 20 min time gap showed a lower percent of the area covered by cells in comparison with 150 rpm. Twenty min time gap is not enough for cells to attach to the bottom of the well plate and elongate. At 200 rpm, most of the attached cells were removed from the surface due to the magnitude of the shear stress (Fig. S5).

It has been demonstrated that oscillatory shear flow implies two main effects on cells. One effect is a flow-velocity-dependent change in mass transport. Nutrition diffusion to the cell surface increases as flow velocity or shear rate increases, leading to further stimulation of cells. Mechanical cell deformation due to shear stress is another effect which has been

studied comprehensively [22]. In this study, we have shown that time gap between cell seeding and applying shear stress is also an important parameter for better cell functions. We have verified that the shear stress generated by an orbital shaker can cause cell migration to the center region of the well plate due to the lower magnitude and fluctuation of the produced shear stress. A higher number of cells were attached after 20 min time gap and before applying shear stress for the specific magnitude of shear stress. Nevertheless, the first limitation of this study was the discrete increment of rotational speed which produced discrete magnitude of shear stress. This limitation did not allow us to find the exact optimum shear stress in the experiment. Another limitation is calculating the exact value of shear stress. Although it has been shown that the used equation has reasonable accuracy, using other methods to find the magnitude of shear stress, such as, using computational fluid dynamic methods and software should be used to verify the results. In this paper, we are able to demonstrate that osteoblasts start developing extracellular components at the early stages of seeding and the time gap is beneficial for increasing number of cell attachment and proliferation. However, this attachment is weak and cells can be easily detached by applying higher magnitude of shear stress (i.e., 200 rpm). This study is one of the first studies that considers time gap between cell seeding and applying shear stress and investigating cell biological properties. DNA content would be beneficial to elucidate the mechanism behind developing cell attachment proteins and extracellular matrix. In addition, the effect of other parameters such as cell number and cell type can be investigated. The results of this study is useful for designing new osteoblast bioreactors for scaffold based and scaffold-free in the base bone tissue engineering.

Conclusions

Our results have shown that similar to endothelial cell lines, osteoblasts are also sensitive to the mechanical stimulation. Small change in the magnitude of shear stress can greatly affect the cell response. We have shown that time gap between cell seeding and applying mechanical stimulation also affects the cells reaction. Waiting for 20 min before applying shear stress increased the number of attached cells in all different rotational speed. In addition, cells do not respond to the low shear stress as seen at the speed of 50 rpm. This suggests that 50 rpm may be a lower limit of rotational speed needed to induce a cellular response. This, however, would have to be confirmed with additional testing. On the other hand, applying high magnitude of shear stress as we did in 200 rpm, is not beneficial for cell attachment and elongation. In summary, mechanical stimulation improves cell attachment and proliferation for osteoblasts. However, rotational speed and time gap between cell seeding and mechanical stimulation should be optimized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Effect of oscillatory mechanical stimulation on osteoblast functions was studied.
- Cells were exposed at three levels of speed with or without waiting for 20 min to attach cells.
- Shear stress magnitude and time gap allowing cells to attach are important for cell functions.
- Cells start developing extracellular components at the early stages of seeding.

Fig. 1. Dividing well to different areas for Live/Dead cell assay

A well in the culture plate is divided to different parts to study the effect of experimental parameters.

Fig. 2. Cell attachment and spreading on different regions of the well plate, (A) at edge, (B) at periphery and (C) center, at 50 rpm with 20 min time gap when cultured at different time points.

Fig. 3.

The effect of shear stress on (A) total percentage of area of the wells in the plate covered by cells, (B) percentage of area covered by cells in different parts of the well plate, at 50 rpm with 20 min time gap, respectively. (C) Total percentage of area of the wells in the plate covered by cells, (D) percentage of area covered by cells in different parts of the well plate at 50 rpm without time gap. * denotes the significant difference from control samples.

Fig. 4.

The effect of shear stress on (A) total percentage of area of the wells in the plate covered by cells, (B) percentage of area covered by cells in different parts of the well plate, at 100 rpm with 20 min time gap, respectively. (C) Total percentage of area of the wells in the plate covered by cells, (D) percentage of area covered by cells in different parts of the well plate at 100 rpm without time gap. * denotes the significant difference from control samples.

Fig. 5.

The effect of shear stress on (A) total percentage of area of the wells in the plate covered by cells, (B) percentage of area covered by cells in different parts of the well plate, at 150 rpm with 20 min time gap, respectively. (C) Total percentage of area of the wells in the plate covered by cells, (D) percentage of area covered by cells in different parts of the well plate at 150 rpm without time gap. * denotes the significant difference from control samples.

Fig. 6.

The effect of shear stress on (A) total percentage of area of the wells in the plate covered by cells, (B) percentage of area covered by cells in different parts of the well plate at 200 rpm with 20 min time gap. * denotes the significant difference from control samples.