

Research Article

Studies on a Novel Bioreactor Design for Chondrocyte Culture

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Received 8 April 2013; Revised 17 June 2013; Accepted 7 August 2013

Academic Editor: Vassilios I. Sikavitsas

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A bioreactor system plays an important role in tissue engineering and enables reproduction and controlled changes in the environmental factor. The bioreactor provides technical means to perform controlled processes in safe and reduced reproducible generation of time. Cartilage cells were grown *in vitro* by mimicking the *in vivo* condition. The basic unit of cartilage, that is, chondrocyte, requires sufficient shear, strain, and hydrodynamic pressure for regular growth as it is nonvascular tissue. An attempt has been made to design a novel airlift reactor for chondrocyte culture, and the reactor has been evaluated for its performance. The design includes internal loop wavy riser airlift reactor for chondrocyte culture with 5% CO₂ sparging which gives a good yield of chondrocyte after 28 days. The wavy riser provides more surfaces for collision of fluid flow so to create the turbulence. Also, the horizontal semicircular baffles create an angle of 180° which helps in high shear rate. The optimized L/D ratio of the designed airlift reactor (for chondrocyte culture) is 5.67, and it also exhibits good mixing performance.

1. Introduction

Tissue engineering has opened new strategies for the study and growth of tissue *in vitro*. It has the potential of improving the quality of life by repair and regeneration of these tissues in people with traumatic injuries and arthritis. The goal has been directed towards the development of cell line to restore, maintain, or improve tissue function [1, 2].

Bioreactors represent an attractive tool to accelerate the biochemical and mechanical properties of the engineered tissues providing adequate mass transfer and physical stimuli. Various bioreactors have been developed for tissue development during the last decades based on different physical stimulation concepts [3].

The product/process for each type of tissue is to be viewed as a simple bioprocess which includes cell sourcing, construction technique, bioreactor growth, and harvesting process. An integration of these components is required for the successful production of engineered cartilage [4]. The cartilage is composed of chondrocyte and proteoglycan that primarily have GAG and type II collagen. The chondrocyte repair *in vivo* is a slow process [5]. Inadequate cartilage repair *in vivo* has involved the developments of alternative therapies

to restore time and function. Cartilage tissue engineering bioreactors play a crucial role in the final properties of engineered cartilage [6]. The environment of the bioreactor directly affects the uniformity of cell seeding into three-dimensional scaffolds as well as the maintenance of the chondrocyte phenotype and, therefore, the characteristics of the cartilage tissue [7]. Bioreactors are responsible for providing the right mass transfer conditions for nutrients and waste to be exchanged between the culture medium and the chondrocytes and suitable mechanical induction for the cartilage tissue production [8]. Ideally, the bioreactor itself provides *in vivo*-like physical stimulation to the growing tissues, either by mechanical or hydrodynamic loading, enhancing the secretion of extracellular matrix and tissue formation [9].

Various bioreactors have been developed and used for culturing chondrocyte and tissue engineering constitutes, which are allowed to be grown under variable hydrodynamic environments [10]. The design variation in bioreactors provides a different hydrodynamic environment, which results in variations in tissue properties. Differences in flow and mixing conditions around the growing chondrocytes/cartilage constructs affect tissue structure by inducing changes in

cell shape and function as well as in mass transfer rates of nutrients and metabolites [11]. The objective of this study was to design a wavy-walled airlift bioreactor (ALR) to enhance mixing at controlled shear stress for *in vitro* chondrocyte culture. The wavy-walled bioreactor might be useful in studies aimed at distinguishing the effects of the hydrodynamic environment for tissue-engineered cartilage.

Characterizing the wavy-walled bioreactor's hydrodynamic environment and its performance for cartilage cell and tissue cultures may help establish direct relationships between hydrodynamic forces and chondrocyte properties. Earlier studies on wavy-walled bioreactor have shown that it enhances mixing at controlled shear stress levels. It has also been suitably suggested that further studies on characterization of hydrodynamic environment effect on the growth of chondrocytes be evaluated in detail [12]. The proposed novel wavy-walled airlift bioreactor was observed to be useful for fundamental studies on chondrocyte growth for cartilage regeneration and to assess the significance of cell density and nutrient and hydrodynamic conditions for development [13].

2. Materials and Methods

2.1. Biopsy Collection and Isolation of Chondrocytes. The articular cartilage isolated from rabbit limb knee joint was harvested under sterile condition by creating defect of 3 mm in lateral condyle of tibia. The cartilage was collected from the Department of Orthopedics and Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India (the study was approved by the Ethical Committee of the Institute). The specimen of rabbit cartilage obtained from the knee of rabbit was processed for chondrocyte isolation. Each specimen (free of perichondrium and other noncartilaginous connective tissue) was washed with Dulbecco's modified Eagle's medium, minced finely, and incubated for 1 h with 1 mg/mL pronase solution at 37°C. Later, the cartilage fragments were again washed with DMEM and incubated with a 200 U/mL collagenase type II solution overnight at 37°C. The obtained cell suspension was filtered through strainer (pore size 80 μ) to remove any undigested tissue and centrifuged at 2600 rpm for 10 min. The supernatant was discarded, and the cell pellet was washed with serum-free DMEM medium. Finally, the cell pellet was resuspended in 5 mL DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 50 μ g/mL gentamycin, 2 μ g/mL amphotericin-B, and 50 μ g/mL L-ascorbic acid to 12 m² tissue culture flask and maintained at 37°C, in a humidified atmosphere under 5% CO₂. The culture medium was replaced twice weekly, and cells were allowed to grow until subconfluence [14].

2.2. Cell Count and Viability. Total cells and percentage of viable cells were counted, soon after cell isolation and during the process of growth, using trypan blue vital dye in a haemocytometer [15].

2.3. MTT Assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-di phenyltetrazolium bromide, a yellow tetrazole) measurements were performed by replacing the DMEM culture

medium with MTT solution. MTT solution was added to each tissue culture well, mixed by tapping gently, and incubated at 37°C for 4 hours. The medium was removed, and DMSO was added into each well to dissolve the formazan by pipetting. The extinction was measured at 490 nm [16], to observe the cell viability.

2.4. Airlift Reactor Design. A novel airlift bioreactor (ALR) of volume 110 cm³, was designed and fabricated of borosilicate glass, Figure 1 [17]. This ALR had a concentric tube (the riser) of diameter 10 mm, and the outer column (downcomer) diameter was 30 mm. Riser was wavy type having sinusoidal wavelength of 30 mm and amplitude of 2.5 mm. The total height of ALR was 230 mm with riser height 150 mm and down comer 170 mm. The top and bottom clearance were maintained at 15 mm and 10 mm, respectively. Baffles were also present on the walls of downcomer, 5 mm wide, semi-circular alternatively placed and 5 mm apart.

To optimize the various length diameter (L/D) ratios for the reactor, the length of column was changed to 200 mm, 230 mm, and 260 mm, whereas the corresponding length of the riser was 120 mm, 150 mm, and 180 mm, respectively. The diameter was maintained constant at 30 mm. The mixing time for all of these ALR was evaluated using a conductivity meter.

The optimized ALR had $A_r/A_d = 0.125$, $A_d/A_c = 0.89$, and aspect ratio (L/D) of 5.67. A_r is the area of riser, A_d is the area of downcomer, and A_c is the area of column. The ALR had a single nozzle type gas sparger made of SS316 steel, at the bottom. The sparger was connected to a pump, to give sterile filtered (5% CO₂ in air) air through the riser. The mixing time evaluation studies and chondrocyte cell growth studies were made for all the three ALR separately.

2.5. Culture of Chondrocyte in ALR. The free chondrocytes cells, isolated from rabbits, were previously suspension cultured in shake flask using DMEM media and then transferred aseptically in the ALR through a side arm tube. This was provided with sterile gas supply (5% CO₂ in air) through the sparger. The sterile (5% CO₂) air was sparged at the bottom of the riser at a controlled flow rate, which caused a decrease in the density of the fluid locally. Hence, the fluid moved up in the riser along with the liquid/pellets, and finally the air bubbles disengaged at the top of the ALR. The liquid moved down through the downcomer into circulation cycle. This provided the mixing and mass transfer in the cell growth process.

The chondrocyte cells were grown in the novel ALR for 28 days, and the nutrition media were replaced every week with the depletion of nutrient. The growth of chondrocytes was observed for changes in parameters like pH and glucose level.

2.6. GAG Release Quantification. Total sulphated glycosaminoglycan (GAG) content in the chondrocyte culture in ALR was assayed in triplicate, using 1 mL 1,9-dimethyl methylene blue solution and aliquots of 25 μ L trypsin digest solution. The optical density was determined at 595 nm. Standard curve was established by chondroitin sulphate (CS)

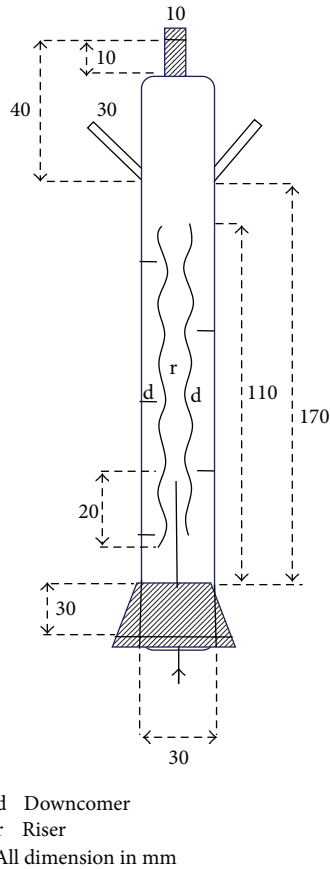


FIGURE 1: Schematic representations of novel airlift bioreactor.

serial dilutions of 0–100 $\mu\text{g/mL}$. The samples were taken at equal time intervals, and optical density was measured at 595 nm and compared with standard curve [18]. Cell collagen content was determined by hydroxyproline quantification after acid hydrolysis (6 N HCl for 3 hrs at 130°C). A multiplication factor of 9 was used to evaluate collagen content from hydroxyproline values [19].

2.7. Total DNA Content Evaluation. The total DNA content of the chondrocyte culture was determined using the Hoechst method. Fluorescence was measured with 355 nm excitation filter and emission at 460 nm [20].

2.8. Mixing Time Measurement. 50 mL of saturated solution of NaCl was injected (nearly ideal impulse) at the top of downcomer. The tracer was sampled at 5 cm from the gas distributor in the down comer. The conductivity was analyzed by conductivity meter. All the experiment was carried out under atmospheric pressure and ambient temperature of 25°C. Tap water and air are used as the working fluids. To verify the laminar flow in the bioreactor (without cells), plasticine spheres suspended in PBS were visualized to follow steady path time during aeration at defined rates in ALR. The viscosity of the water and the DMEM media was observed to be approximately the same; that is, distilled water is 1.0 and DMEM is 1.1, and studies were made in triplicate.

2.9. Media Analysis. Samples were withdrawn before and after each feeding (i.e., after every 07 days) during the growth duration of 28 days of study. Glucose and lactate levels were determined using biochemical kits. Also, the pH variation during the bioreactor growth was monitored regularly.

2.10. Statistical Analysis. All the results were expressed as the mean of at least three separate experiments and analyzed statistically. The standard error was evaluated and plotted in the profiles, and P value was calculated. The statistical significance was accepted at $P < 0.05$.

3. Results and Discussions

3.1. Effect of L/D Ratio on Mixing Time. The liquid mixing time is an important hydrodynamic characteristic of airlift reactor design. The different airlift reactors having different L/D ratios were used for the mixing time measurement. The observations for the varying mixing time for reactors having L/D ratios 4.67, 5.67, and 6.67 were 6.8 sec, 9.2 sec, and 11.1 sec, respectively, as shown in Figure 2. This depicts the optimized mixing time of 9.2 sec for the designed L/D of 5.67 for the proposed ALR, which was observed to be the optimal for chondrocyte culture. The observation of decrease in mixing time with the decrease of L/D ratio is quite similar to the earlier studies [21]. Further, the effect of mixing time on cell hydrodynamics may be evaluated using computational fluid dynamics (CFD) studies.

3.2. Effect of L/D Ratio of ALR on Chondrocyte Growth. All three airlift reactors of different L/D ratios were used for initial chondrocyte culture for 28 days. The cell count of each reactor was measured as shown in Figure 3. The observations were in triplicate, and the mean values were calculated. The cell count was different for different L/D ratios of reactor. It was observed that L/D of 5.67 was good for chondrocyte growth than other reactor dimensions and corresponded with the mixing time of 9.2 sec.

3.3. Hydrodynamic Studies. Before data collection, aeration of reactor was carried out over a long period of time to ensure that steady state flow distribution was established in airlift reactor, and experiments were carried out to operate with complete gas disengagement. The reactor was always operated in homogeneous flow (at superficial gas flow of less than 0.08 m/s), on the basis of visual observation. The fluid flow inside the novel ALR was due to the pressure difference at the bottom of the ALR caused due to air sparging.

The pressure difference is calculated by

$$\Delta P = \rho L g (\phi_r - \phi_d), \quad (1)$$

where ΔP is pressure difference, ρL is density of liquid, ϕ_r and ϕ_d are gas hold-ups of riser and downcomer. It acts as the driving force for the liquid movement.

The optimized airlift bioreactor ($H/D = 5.67$) was operated under various air flow conditions of 0.03 m/s, 0.04 m/s, 0.05 m/s, 0.06 m/s, and 0.07 m/s superficial gas flow rate.

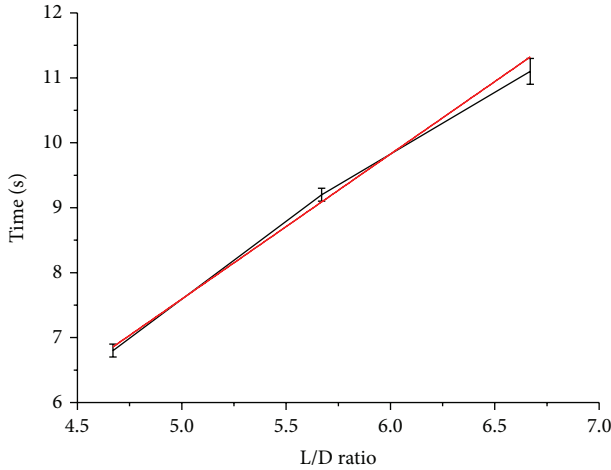


FIGURE 2: Mixing time for different L/D ratio ALR which shows linear fitting of experimental data with P value 0.032 and R^2 as 0.99.

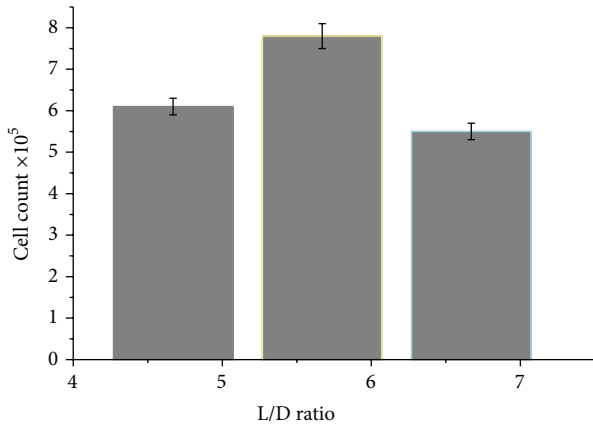


FIGURE 3: Cell count variation in ALR of different L/D ratios.

The Reynolds numbers for aeration rate were observed to be 818.18, 1090.91, 1363.64, 1636.36, and 1909.09 for 0.03 m/s, 0.04 m/s, 0.05 m/s, 0.06 m/s, and 0.07 m/s, respectively, and were observed to be well below the stability requirement of $N_{Re} < 5000$. To ensure steady one-dimensional flow, vortex for aeration in this geometry, sterile gas (5% CO_2) flow was supplied for the chondrocyte growth, using a nozzle.

The liquid circulated in the ALR was due to the sparging of air. Fluid flow was usually in straight direction with the sparged air, but due to wavy nature of riser the flowing fluid collides with the walls of riser, that is, fluid reflects at angle of collision and also in reverse flow vector. The fluid collision with the wavy wall created turbulence. Similarly, the returning fluid also caused turbulence due to the baffles. The baffles and wave might create an angle of 180° which caused high shear force. Chondrocyte culture requires a superficially high shear force as well as the hydrodynamic pressure which was created by fluid inside reactor, to support the growing chondrocytes. The shear stress governed by the liquid of each region can be defined as the energy dissipated divided by the mean path of circulation in the region and by the sum

TABLE 1: Shear stress in ALR.

| Superficial gas velocity | 0.03 m/s | 0.05 m/s | 0.07 m/s |
|---|------------|-------------|-----------|
| Average shear stress (MPa) | 10.85 | 13.79 | 16.03 |
| Range of shear stress (MPa) | 3.67–46.29 | 6.13–59.367 | 8.6–69.23 |
| Cell count ($\times 10^5$) at 28 days | 7.5 | 8.6 | 7.4 |

of the areas of bubbles. The viscosity for the growth media was observed to increase with the growth and was maintained with the replacement of fresh medium every 7 days. The shear rate for ALR due to air sparging may be calculated by the following:

$$\Gamma = 3.26 - 3.51 \times 10^2 U_{gr} + 1.48 \times 10^4 U_{gr}^2, \quad (2)$$

where P_G is power input due to gas (W), V_L is volume of liquid inside the reactor (m^3), and U_{gr} is the superficial velocity of gas.

The average shear stress was observed to be 13.79 (MPa) whereas the overall shear rate for the proposed ALR was observed as 22.71 sec^{-1} , at $U_{gr} 0.05 \text{ ms}^{-1}$ and maximum cell mass at 28 days. However, the variation in the shear stress with the superficial gas velocity is defined in Table 1.

Equation (2) has been developed in response to the viscosity associated reduction in the liquid circulation velocity in the cell culture broth in laminar flow regime. Although the hydrodynamic environment in various zones of airlift reactor tends to be quite different, for a small reactor size, the characterization of global shear rate in bulk flow may be done by the previous equation.

Also, the overall shear rate may be deceptively low, even though the damaging levels may be experienced in high shear zones [22]. The identified specific energy dissipation rate increases in the order downcomer < riser < bottom, for any fixed value of aeration [23].

3.4. Cell Count and Cell Viability. Experiments were conducted to evaluate chondrocyte growth and cartilage development in shake flask and ALR, with reference to the effect of hydrodynamic loading on chondrocyte proliferation. It was observed that in ALR, higher cell density was obtained which suggests that the flow in the bioreactor stimulates chondrocyte proliferation. Chondrocyte cultures were grown for 28 days. Growing cells were quantified using hemocytometer and trypan blue dye to analyze the cell number per milliliter as well as their viability. Isolated cells were cultured and compared using growth media in shake flask as well as in ALR. The growth was evaluated by intermittent sampling during cell culture on the 1st, 2nd, 7th, 14th, 21st, and 28th days of the cultures. Chondrocyte cells were observed to exhibit rounded morphology for 28 days of growth which is a characteristic of these cells. The observations were statistically evaluated, and the results are shown in Figure 4. The variations in the values were within limits.

The MTT measurement was performed to evaluate the cell viability of chondrocytes grown in shake flask and ALR. The MTT assay showed the metabolism of the tetrazolium salt MTS into formazan by viable cells. The extinction was

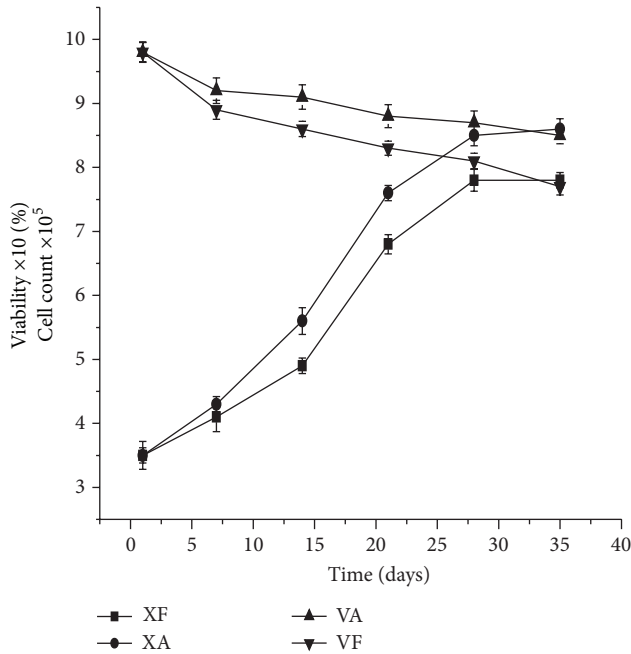


FIGURE 4: Comparison of viability and cell count in ALR and shake flask.

measured at 490 nm. The variation of the absorbance over the period of growth was correlated to the cell growth, observed maximum at 28 days. The growth of cells in ALR was better than in shake flask.

Figure 4 shows the variation of the cell count (XF (Flask), XA (ALR)), and viability (VF (Flask), VA (ALR)) with reference to time. It can be observed that the average cell viability is higher for ALR (89%) compared to the shake flask which is less than 80%. Also, the level of cell count for ALR is quite higher, about 12–15%, than that for shake flask grown cells.

3.5. GAG Quantification. The total GAG content of the grown chondrocytes in shake flask and ALR was evaluated at various time intervals and statistically analyzed. The results are shown in Figure 5. It can be observed that the GAG level in the ALR higher than that in shake flask might be due to mechanical shear on the chondrocyte which probably increased the GAG content in chondrocyte cells. Also, the cumulative soluble GAG production generally increased in ALR compared to shake flask. GAG concentration reached a maximum at 48 mg/L in airlift reactor in 28 days. These observations may be compared to similar studies on GAG using culture of articular chondrocytes in hydrogel constructs in Rotary wall vessel which showed lower GAG level at 25 mg/L, in 20 days [1].

It can be suggested that *in vitro* culture of chondrocyte and the applied shear stress may stimulate ECM synthesis and GAG deposition in the cells, as observed in the wavy ALR. The GAG content and collagen level increase during the growth of chondrocyte in bioreactor as shown in Table 2. However, the collagen content per unit GAG content was the

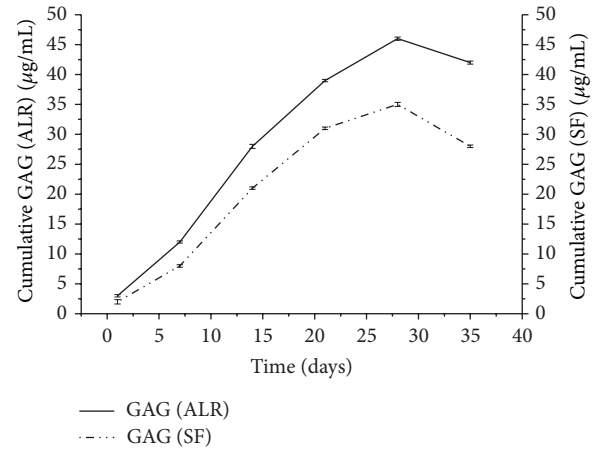


FIGURE 5: Cumulative GAG content in chondrocytes versus time (days).

TABLE 2: Glucose/lactate Balance.

| | 2-week growth time | 4-week growth time |
|------------------------------------|--------------------|--------------------|
| Total GAG ($\mu\text{g/mL}$) | 28 | 45 |
| Collagen/cell ($\times 10^{-7}$) | 1.6 | 2.6 |
| Collagen/GAG ($\times 10^{-9}$) | 5.71 | 5.77 |
| Lactate/glucose (L/G) | 0.95 | 1.6 |

highest after 14 days but decreased later at the end of 28 days. These results were similar to the earlier observations [24].

3.6. Glucose/Lactate Balance. Nutrient utilization was evaluated during cell growth to assess the cell growth environment. It was observed that the average glucose consumption and lactate production rate were slightly high for ALR compared to shake flask, but these levels were within limit as shown in Table 2.

During the growth study, it was also observed that, as the growth proceeded, the L/G ratio changed. In the first week, the L/G ratio was less than 1.0, which increased to more than 1.0, with increasing growth. This depicts that in early phase aerobic environment supports the growth which further shifts to anaerobic mode in the late stages.

Lactate/glucose ratio (L/G) was observed to be 1.6 after 4 weeks (studied in triplicate) for the airlift reactor study, indicating an environment where both aerobic and anaerobic pathways were utilized. These observations were quite similar to the previous study [25].

To study and optimize the chondrocyte growth and cartilage production for tissue regeneration and to identify the factors that stimulate growth, attempts were made to design protocol that put the study to uniform conditions in time and space.

A novel designed ALR has been used to improve the growth of the engineered cartilage *in vitro*. Large amount of cartilage growth was observed in the novel ALR. It was further observed that ALR with wavy-walled riser enhanced nutrient transfer, exhibiting the hydrodynamic environment which induces extra shear stress to promote the synthesis of

chondrocyte matrix. This extra shear generated in the ALR simulated the conditions of mechanical load on the growing cells. The mechanical force is playing a fundamental role in the regeneration of cell function, including the induction of gene expression, protein synthesis, cell growth, and differentiation [26]. There may be one of these mechanotransduction mechanisms during cell growth, which were responsible for enhanced chondrocyte growth in ALR [27]. Significant observations have supported that hydrodynamic forces affect the rate of DNA synthesis and relative proportions of the cell in the various cell cycle stages. The cell propagation might be influenced upto 45% by agitation and gas sparging.

While growing the cartilage in the ALR, the pH and glucose level depletion were observed. The pH values of the bioreactor were maintained between 6.9 and 7.2. During the growth, chondrocytes experienced variation in the nutrient level, and then media were replenished periodically so that no nutrient limitation was observed. At the end of the first week, the rounded cells occupied the bottom of the flask, and after three weeks, chondrocyte exhibited spindle shape. However, in ALR, for four weeks of cultivation, the cells remained circular, rounded, and polygonal due to applied shear stress. The cell count in ALR and shake flask has been compared and shown in Figure 5, where it is observed that ALR shows higher growth with 8×10^5 cells. Similar studies have been conducted by Saini and Wick [24] in concentric cylinder bioreactor during production of tissue engineering of cartilage and observed the highest cell density of 15×10^6 cells after 28 days. Also, the pH is observed fluctuating between the 6.78 ± 0.11 . This fluctuation is attributed to nutrient depletion and to release of acidic metabolite (lactic acid) in media.

It is also important to mention that the articular cartilage of the major weight bearing joints in the hip (human) and the knee has average loadings of about 0.5–7.7 MPa, when the same joints were subjected to activities like repeated knee bending or stair climbing and exhibit maximum values of shear 18 MPa (during walking and knee bending). Also, the designed ALR, with Usg of air at 0.05 ms^{-1} , exhibit maximum shear of $22.71 \text{ sec}^{-1} \text{ MPa}$, which may be supportive of chondrocyte growth as observed. These studies also support that shear and compression enhance chondrocytes GAG and collagen synthesis.

4. Conclusions

An important issue for cartilage bioprocessing is the development of bioreactor that may produce uniform tissue. As observed in this study, the wavy-walled concentric ALR provides the *in vivo* condition for the chondrocyte growth. It was observed that the rate of chondrocyte growth increased because of extra shear and good mass transfer as observed in the ALR. The shear rate and the hydrodynamic forces inside the reactor are increased due to the baffles and the wavy nature of the riser which also helps to maintain high Reynolds number, so the nutrient transfer is increased and results in good chondrocyte growth. This study has demonstrated that the designed wavy wall ALR provided a culture environment

for chondrocyte growth with enhanced GAG and collagen content, which may be attributed to the homogenous hydrodynamics and mass transfer environment prevalent in the bioreactor. The observations have revealed that chondrocyte morphology was well preserved in all bioreactors (data not shown) used in the study. Also, the cultured chondrocyte in ALR exhibits enhanced GAG contents in the cell probably due to enhanced cell-cell interaction as the tensile stress may be a crucial factor for modulating the maturation of chondrocytes. Further, attempts may be made to upscale the technology for large scale production of chondrocytes to be used for cartilage tissue engineering and also evaluation of hydrodynamics of cell culture using CFD techniques.

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