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A Perfusion Bioreactor for Long-Term Studies of the Dynamics of the Formation of a Tissue Equivalent

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This paper presents results obtained from a modified perfusion bioreactor designed to form tissue equivalents in conditions of long-term cultivation with the capacity to oxygenate the medium. The design of the device permits the morphology of cellular engineering structures to be studies at four cultivation time points under flow conditions and also provides for constant monitoring of culture medium pH, O_2 and CO_2 contents, and major metabolite contents throughout the experiment. The functional effectiveness of the bioreactor is confirmed by the example of "growing" the tissue equivalent of cartilage for 25 days with maintenance of cell culture viability, with constant values for the main culture medium parameters and sterility.

Introduction

All organs within the body are exposed to biomechanical forces such as directional compression, stretching, shear forces, hydrostatic pressure, the set of influences being unique to each organ [1]. Thus, creation of functionally active cellular engineering structures (CES), whose main elements are cells and cell carriers (matrixes, scaffolds), requires the most accurate possible reproduction of not only biochemical, but also biomechanical stimuli which function to ensure the vital activity of cells [2-4]. Special devices – bioreactors – are used to simulate physical effects in vitro [4-6].

Stable and efficient operation requires the bioreactor to meet the following requirements: its components must be biocompatible and corrosion-resistant, hermetically sealed, compatible with a variety of cell carriers, and economical in terms of consumption of culture media; it must operate in flow mode and provide continuous monitoring of cultivation conditions. Furthermore, study of the dynamics of the formation tissue equivalents during cultivation in the bioreactor requires independent removal of CES from the circulation system. Another important aspect is the ability to run long-term experiments, which is associated with a high risk of contamination.

The past decade has seen the development of advanced bioreactors able to maintain stable and reproducible conditions corresponding to in vivo physiological cell niches. Note that increases in the complexity of a system bring conditions closer to the natural, while simplification makes the device more reliable. In this regard, the task of finding a compromise between the simplicity of the bioreactor and the functional parameters of the desired final product is important [7]. One of the most suitable study systems for validating bioreactor design is the chondrogenic differentiation of mesenchymal stromal cells (MSC), as this is a long process and mechanotransduction plays an important role in the formation of the normal chondrocyte phenotype [8, 9]. A previously developed perfusion bioreactor allowed cultivation for up to 14 days with four parallel CES specimens but did not provide independent removal of CES from the circulation system or monitoring of the culture medium parameters [10]. However, cultivation for more than 14 days involves multiple culture medium changes, increasing the risk of contamination and ingress of air into the pipelines.

The aim of the present work was to modify the design of an existing perfusion bioreactor to run long-term studies with the option of oxygenating the culture medium and replacing it without contact with air.

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Materials and Methods

Figure, 1, a shows the modified version of the perfusion bioreactor. The significant advantages over the prototype [10] are: provision of O_2, CO_2, and pH sensors, a sampling unit, and an oxygenator connected to a cylinder with a gas mix. The number of tanks with expensive culture media was reduced without reducing the number of parallel culture chambers in the circulation system. The sampling unit allows culture medium samples to be examined and replaced under sterile conditions. The culture chambers and the tank containing the medium were placed in an incubator where a temperature of 37°C and a relative humidity of 90-100% were maintained, along with a CO_2 content of 5% and an O_2 level of 20%.

A Hemofilter D150 oxygenator (Medica S.p.a., Italy) was positioned behind the unit connecting the two culture chambers in the direction of the flow of the medium and was connected to a cylinder containing the gas mix and the atmosphere via sterile filters. The following were installed in series on the parallel lines: a pressure sensor (PendoTECH, USA), an O_2 sensor, a CO_2 sensor, and a pH sensor (all from Polestar Technologies, USA), and a sampler. A stand holding the culture chambers was developed to hold the bioreactor elements in the incubator and facilitate access to the culture chambers and culture medium tank (Fig. 1, b).

The functional effectiveness of the modified bioreactor was studied in an experiment on chondrogenic differentiation of MSC from human adipose tissue (MSC ATh) as part of the CES. Passage 3 cells were isolated and cultured in complete growth medium (CGM) for experiments using standard methods [10]. The matrix was a biopolymer microheterogeneous collagen-containing hydrogel (CCHG) (Sphero®GEL, BioMIR Servis, Russia). Previous studies have demonstrated the ability of CCHG to maintain the chondrogenic differentiation of MSC ATh [11]. TGF-β1 (PeproTech, USA) was used to induce chondrogenic differentiation. The medium was replaced every 7 days.

The protocol for preparing cartilage tissue equivalent consisted of several stages. On day 1, all CES contained 1 mL of CCHG and $6 \cdot 10^6$ MSC ATh in 0.5 mL of CGM and were placed in the culture chamber of the bioreactor and incubated for one day without flow. On day 2, the chamber containing the CES was placed in the bioreactor circulation system at a flow rate of 1.0 mL/min. On day 4, the medium was replaced with differentiation medium. On days 4, 11, 18, and 25, CES were removed from the bioreactor and samples of culture fluid were collected for monitoring for microbial contamination using a standard method consisting of inoculation of trypticase-spy broth. Glucose was estimated on days 18 and 25, using a Konelab Prime 60i analyzer (Thermo Fisher Scientific, Finland).

Sample morphology was assessed by staining with hematoxylin and eosin and Alcian blue. Cell viability was determined by fluorescent staining with Calcein AM (Life Technologies, USA).

Data were analyzed statistically in Microsoft Excel. Significant differences were identified using Student's t test at $p < 0.05$.

Results

The modified perfusion bioreactor including four independent culture chambers filled with model solution

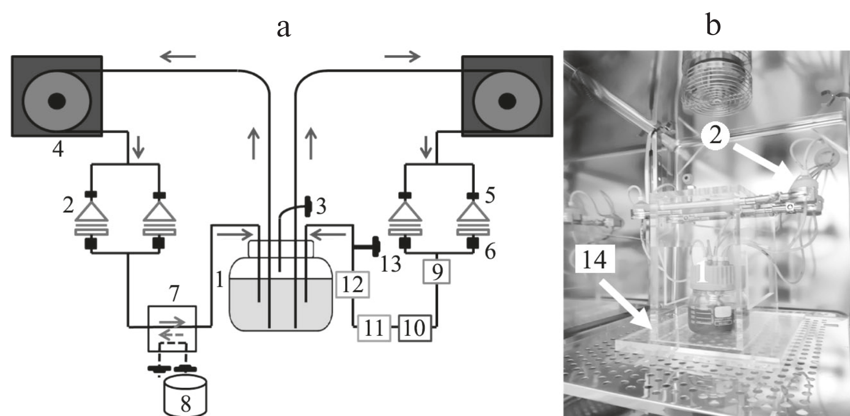

Fig. 1. Perfusion bioreactor: a) circulation system; b) stand for attachment of culture chambers; 1) culture medium tank; 2) culture chamber; 3) sterile filter; 4) peristaltic pump; 5, 6) connectors; 7) oxygenator; 8) cylinder containing gas mix; 9) pressure sensor; 10) O_2 sensor; 11) CO_2 sensor; 12) pH sensor; 13) sampling unit; 14) stand for attachment of culture chambers.

TABLE 1. O_2 and CO_2 concentrations and pH in the bioreactor system before and after saturation of the liquid with a gas mix containing 5% CO_2

Description of medium in bioreactor circulation system	O_2, %	CO_2, %	pH
Water	20.4 ± 0.3	0.0	6.37 ± 0.01
Water + saturation of liquid with gas mix containing 5% CO_2	21.2 ± 0.2	4.9 ± 0.1	6.34 ± 0.01
DMEM culture medium	17.8 ± 0.5	0.0	7.84 ± 0.03
DMEM culture medium + saturation of liquid with gas mix containing 5% CO_2	20.5 ± 0.3	5.1 ± 0.1	7.62 ± 0.01

was tested. The aim was to confirm the ability of the bioreactor to oxygenate the medium and to monitor culture medium O_2, CO_2, and pH.

Table 1 shows the effectiveness of the culture medium gas saturation unit and the O_2, CO_2, and pH sensors in the bioreactor circulation system on filling with model solution (water) and Dulbecco growth medium. We note that gas saturation of the culture medium is critical for certain cell cultures: for example, hyperoxygenation (40% O_2) increased the activity of the urea cycle and cytochrome P450, bile acid synthesis, and ammonia release by 165-266% in HepaRG human hepatoma cells [12].

Experiments addressing the formation of cartilage tissue equivalent did not use additional gas saturation of the culture medium as chondrogenic differentiation of MSC was run in standard culture conditions (5% CO_2, 20% O_2) [13]. After the first day of the experiment, cells were attached to the CCHG and spread out (Fig. 2).

Staining with Calcein AM demonstrated the presence of live cells on surfaces of the CCHG in all CES (Fig. 2, a, c, e, g, i). On day 4 of cultivation in CGM, the cell mass on the hydrogel surface was minor (Fig. 2, b, d). Cell growth was observed after replacement of CGM with differentiation medium (Fig. 2, f, h, j), with active production of extracellular matrix (ECM) by day 25, increasing both proliferative and functional activity (Fig. 2, j). Preparations also contained cells with cavity-type structures, i.e., young chondrocytes (Fig. 2, j). These cells were located on the surface and in the volume of the matrix. Staining for glycosaminoglycans (GAG) became positive on day 11. Processing of images of histological sections (n = 10) in ImageJ (MIH, USA) was used to determine the ratio of ECM positively stained for GAG to the cell count (CC). The activity of GAG production by cells increased from day 18 to day 25 (107 ± 24 and 224 ± 65 μm^2/CC respectively), indicating differentiation of MSC ATh along the chondrogenic pathway.

Monitoring culture medium pH is an important and informative parameter. The accumulation of metabolites, for example lactate, and the use of serum-free media lead to decreases in pH [14]. Changes in pH can point to contamination. Culture medium pH during the experiment did not undergo any significant changes and was within the range 7.5 ± 0.5; O_2 and CO_2 contents were also con-

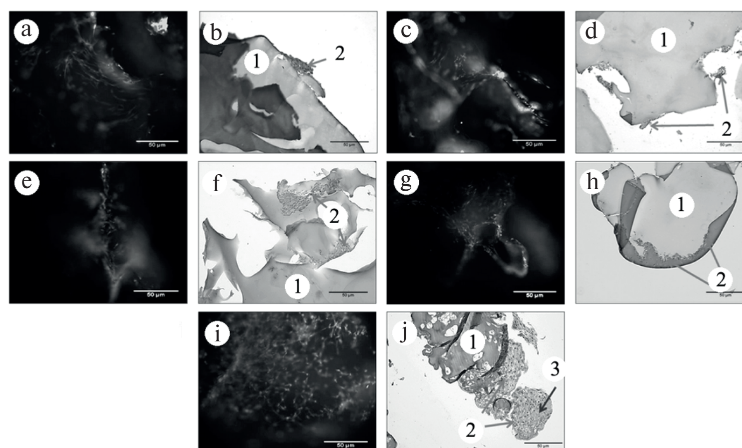

Fig. 2. Dynamics of chondrogenic differentiation of MSC ATh cultured on CCHG in flow conditions at a rate of 1.0 mL/min: staining with fluorescent dye Calcein AM (a, c, d, g, i); staining with hematoxylin and eosin (b, g, f, h, j); day 1 (a, b); day 4 (c, d); day 11 (e, f); day 18 (g, h); day 25 (i, j); 1) CCHG; 2) cells; 3) microsphere-like structures. Scale bar: 50 µm.

stant (19% and 0%). The glucose content of the medium was an indicator of the level of cell metabolism and indicated the need for changes of culture medium. Glucose level, were constant: 24 ± 1 mM on day 18 and 23 ± 1 mM on day 25.

Microbiological studies of samples of medium confirmed that the bioreactor design was able to prevent entry of bacteria into the culture medium.

On culture day 21, MSC ATh displayed high proliferative activity, acquired chondrocyte-typical morphology, and synthesized GAG-containing ECM, which is evidence of the formation of cartilage tissue equivalent. Furthermore, the present study is a validation of the bioreactor and demonstrated that it can be used in long-term experiments (at least 25 days) for "growing" four CES in identical conditions with the option for independent removal.

Conclusions

Thus, these studies show that the modified bioreactor supports long-term (at last 25 days) flow cultivation and independent analysis of the processes forming tissue equivalents in four culture chambers with retention of sterility and monitoring of parameters such as pH, gas concentrations, and major metabolite levels.

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