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CHARACTERISTICS OF SPHEROIDS FROM STEM CELLS OF DIFFERENT SOURCES RECAPITULATING CARTILAGE TISSUE

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ABSTRACT

In this work, which compiles a set of results attained at our laboratories in the last years, we compare spheroids obtained with different types of stem cells to produce biomaterials applicable in the area of cartilage tissue engineering. Mesenchymal stromal cells were collected from adipose tissue, dental pulp, bone marrow, embryonic human tissue and amniotic fluid. A microwell array developed at our laboratories using a micro-mold-based technology was used to produce the spheroids. The cell aggregation behavior, the mean spheroid diameter, sphericity, solidity and size distribution were compared, as well as the capability of the cells in the spheroids to differentiate in micro-tissues recapitulating important features of cartilage. The expected costs of the implementation and use of the methodology employed to produce the spheroids were estimated, indicating that the selected approach to produce the cell aggregates it not only technically feasible, but also highly reproducible and economically competitive.

Keywords: Cell spheroids. Stem cells. Tissue engineering. Cost analysis.

1 INTRODUCTION

Stem cells spheroids are three-dimensional aggregates of stem cells that can mimic the natural 3D environment of tissues, offering advantages such as improved cell-cell interactions and extracellular matrix (ECM) formation, and more accurate replication of *in vivo* features than attained with traditional bidimensional cell culture[1].

These advantages are particularly attractive when concerning application both in lesion repair through cell therapy and tissue engineering, and well as in *in vitro* model tissues to study disease mechanisms and drug testing.

The authors of this work have been exploring the production of spheroids by different techniques in the last decade, for instance, using the classical micromass culture approach[2]. More recently, our team has employed a technique based on the inoculation of different types of stem cells into a micro-molded non-adhesive hydrogel, in the form of a microwell array casted from a 3D-printed primer mold produced with acrylic resin[3-10] . The cells spontaneously aggregate in the microwells environment and form, after a few days, spherical aggregates.

In this work, we discuss a set of results[3-10] spheroids produced from different stem cell types using the developed micro-mold aiming at cartilage tissue engineering application and also analyze the expected costs of implementing and using this methodology.

2 MATERIAL & METHODS

The microwell array micromolded using a non-adhesive hydrogel with previously defined well geometry, height, diameter and spacing was obtained as described previously[3,6].

Different types of cells, including human mesenchymal stromal cells (MSC) from dental pulp (DP-MSC), aminiotic fluid (AF-MSC), adipose-derived (AD-MSC) and fluorescent human embryonic stem cells (hESC) were cultured in culture media appropriate for each cell type, at 37 °C and 5% CO2 for up to 7 days to form the spheroids. The cell aggregates were characterized by different techniques[3-5,7-9]. Cell proliferation, viability and morphology were assessed by microscopy and fluorescence analysis. Spheroid geometric characteristics were analyzed through optical microscopy, with the aid of the ImageJ software. Histological analysis was performed with use of hematoxicilin & eosin, Masson's trichrome, Picrosirius red and Alcian blue staining procedures, and when applicable, the quantification of collagen I, II, IX, X, XI and aggrecans was done using imunohistochemical staining.

Cryopreservation of DP-MSC spheroids was also evaluated. Two different sizes of spheroids were tested by depositing either 0.5×10^6 or 1×10^6 DPSCs in the non-adhesive mold[6]. After 5 days of incubation, the spheroids were characterized in terms of their morphology, ECM composition (histological and immunohistochemical analysis), and cell viability to determine the most suitable spheroid size (1×10^6 cells/mold). The efficiency of two different cryopreservation solutions (alpha-MEM/FBS/DMSO and

FBS/DMSO) was assessed through fusion activity and cellular viability using fluorescence analysis[11].The costs of implementation and use of the micro-molded non-adhesive hydrogel developed by our team were analyzed in different scenarios[5], representing the goal to produce reproductively amounts of spheroids of the same size range both for laboratory use (around 100 spheroids) and for cell therapy or tissue engineering application (around 5000 spheroids). In all cases, the total cost (C_{total}) was estimated considering direct and indirect costs, as shown in Equation 1:

$$C_{\text{total}} = \sum (C_{\text{device}} + C_{\text{material}} + C_{\text{lab.operation}} + C_{\text{labor}} \quad (1)$$

where C_{device}, $C_{material}$, $C_{lab.operation}$, and C_{labor} are, respectively, the microwell array cost, the costs of cell culture reagents used per device, costs with labor and costs of operating a specialized cell culture facility. The individual elements used to determine each total cost element are detailed in the work authored by DECARLI et al.[5].

3 RESULTS & DISCUSSION

Three-dimensional cellular aggregates, such as spheroids, can replicate the natural microenvironment of tissues and organs more effectively than isolated cells or cells in two-dimensional culture. For the implementation of drug cytotoxicity and efficacy tests, as well as for cell therapy and tissue engineering applications, it is essential to use approaches economically attractive, simple and effective concerning the control and reproducibility of the spheroids characteristics.

The non-adhesive hydrogel microwell array technology used by our team for spheroid formation allowed to obtain highly spherical aggregates of dental pulp stem cells, bone marrow stromal cells, embryonic stem cells, as well from mesenchymal cells collected from adipose tissue and amniotic fluid, with sphericity above 0.75, solidity higher than 0.95.and diameters in the range of around 200 to 500 μm[3,9,10].

The results showed that the cells in the spheroids remained viable and upon chondral differentiation stimuli, primary cartilage-specific proteins, such as F-actin and collagen II (which makes up 40% of articular cartilage dry weight), together with glycosaminoglycans (GAGs), were detected in significant proportions[3,4].

Immunohistochemical analysis showed increased presence of type II collagen and aggrecan, characteristic constituents of hyaline cartilage. The comparative analysis of extracellular matrix composition in spheroids derived from AF-MSCs and DP-MSCs by immunohistochemical methods revealed substantial concentrations of type II collagen and aggrecan. Additionally, other collagens (types I, II, IX, X, and XI) were found in similar quantities[9].

Decreased collagen I expression (characteristic of the surface region of articular cartilage) and increased expression of collagens IV (which binds to proteoglycans) and II were observed for human mesenchymal stromal cells[4]. No change in collagen X (an important marker for hypertrophic chondrocytes, typical of osteoarthritis) expression was noticed, which consists in a very attractive feature considering the application of spheroids for the production of bioprinted, mature structures.

Particularly for human mesenchymal stromal cells[4], the production of chondrogenic constructs with hyaline-like phenotype was attained when the spheroids were combined with a bioprinting hydrogel and incubated with chondrogenic medium for 28 days. Through this work, we presented a new method for post-bioprinting differentiation that can maintain the inherent ability of spheroids to fuse and their remodeling capacity. This fact confirms the viability of the spheroids and enables their use as building blocks in tissue engineering techniques.

AF-MSC spheroids fusion studies demonstrated the potential for producing a neocartilaginous microtissue[10]. As a result of the favorable biochemical and mechanical characteristics exhibited by the spheroids produced with the different cells, the analysis of their incorporation in a thermosensitive hydrogel is in its final phase, aiming at their utilization as a medical treatment for cartilage lesions through intra-articular injection, but the spheroids are also attractive for the production of 3D-bioprinted structures for surgical implantation[3,4].

Histological analysis revealed that larger cell spheroids produced more extracellular matrix, and these components may assist in the cryopreservation of cells. Collagen II and aggrecans were detected in these aggregates, indicating the presence of cartilaginous tissue components. The hypothesis of ECM cryoprotectant capacity was supported by the results of the LIVE/DEAD assay, which showed a thinner layer of dead cells in the outer region of the thawed 1×10^6 cell spheroids. The cryoprotective solution containing only FBS and DMSO resulted in the highest cell viability; however, the fusion capacity of the spheroids still needs to be improved[11].

According to our studies, microwell array systems are then a viable alternative to bioreactor-based approaches for producing stem cell spheroids. Lower exposure of cells to shear stress is an important advantage of the microenvironment provided by the microwells. While bioreactors may still be the best option for large-scale spheroid production, microwell array systems are highly attractive for bench-scale research and personalized medicine, offering high-throughput capabilities[5]. The results of the cost analysis performed considering the cost of qualified labor as USD 100/hour are compiled in Table 1[5].

The estimated final costs per spheroid and the main determinant cost parameters changed with the scale of spheroid production. The approximate cost of each spheroid produced at the smaller scale was around 21 times higher than that observed at the larger scale. For the lower scale, the costs with labor and operation of a specialized cell culture laboratory were the most significant. For the production of 5,000 spheroids, the costs with labor were also the ones with the strongest impact, followed by costs of cell culture reagents. Given that qualified operators are necessary for both situations, cost savings could be achieved through the adoption of automated systems.

Table 1. Fractional contributions of key parameters (spheroid device investment, cell culture reagents, cell culture laboratory, and qualified labor) to the total costs associated with different demands in terms of spheroid production (adapted from DECARLI et al.[5]).

Number of spheroids produced	Final cost fraction (%)				Total cost (USD)	Cost per spheroid (USD)
	C_{device}	C_{material}	$C_{\text{lab.operation}}$	C_{labor}		
100	8.1	1.5	14.0	77.9	384.94	3.85
5,000	15.1	19.9	9.9	55.3	903.70	0.18

4 CONCLUSION

The challenge of reproducibly producing viable spheroids from different cell types—uniform in size, shape, and surface characteristics, capable of satisfactorily differentiating into chondral tissue—using a simple, reliable, and economically competitive technology was attained. The accumulated results of the studies performed so far suggest that spheroids have a high potential use in the development of personalized and more effective treatments for diseases affecting cartilage tissues, as well as for advancing the understanding of these diseases if employed as model chondral tissues.

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