Next Materials 2 (2024) 100108

Contents lists available at ScienceDirect

Next Materials

journal homepage: www.sciencedirect.com/journal/next-materials

Discussion

Bio-jetted human adipose-derived stem cells remain viable

Prasad Sawadkar [a,c], Ferdinand Lali [a,c], Suwan N. Jayasinghe [b,*]

[a] The Griffin Institute - Northwick Park Institute for Medical Research, Northwick Park and St Mark's Hospitals, Y Block, Watford Road, Harrow, Middlesex HA1 3UJ, United Kingdom

[b] BioPhysics Group, Centre for Stem Cells and Regenerative Medicine, and Department of Mechanical Engineering, University College London, Torrington Place, London WC1E 7JE, United Kingdom

[c] Division of Surgery and Interventional Science, University College London, 43-45 Foley St, London W1W 7TY, United Kingdom

A R T I C L E I N F O

Keywords
Aerodynamically assisted bio-jets
Human adipose-derived stem cells (hADSCs)
Viability
Differentiation
Regenerative medicine and biology

A B S T R A C T

Direct cell handling processes are increasingly becoming important as they allow the controlled deposition of living cells, with precision for a vast number of applications, spanning the printing of cells in either 2D/3D for the reconstruction of fully functional tissues to the delivery of therapeutic architectures bearing cells and genes of interest. Architectures reconstructed with such cells etc are most useful as models, for studying a wide range of molecular and cellular behaviours, to the development of personalised medicines. Our previous work demonstrated the ability for aerodynamically assisted bio-jets to process single and multiple cell-bearing suspensions, to whole fertilised embryos. Those studies found that the post-treated cells and embryos were indistinguishable from untreated controls. In the present study the authors further validate this jetting technology for the direct handling of stem cells, by demonstrating their viability post-treatment and their capacity to differentiate in comparison to controls. These studies together with our previous work unveil, aerodynamically assisted bio-jets as a platform biotechnology for the direct handling of a wide range of cells and embryos.

1. Introduction

Research and development carried out over the past 50 years or more, in tissue engineering and regenerative medicine, has come to realise, that reconstructed tissues from harvested and expanded cells from biopsies, pose an overall convenience to both the patients and workflow in studying molecular, cellular, and tissue functionality. These studies could range from using such functional 3D constructs for testing drugs and understanding their side effects if any, to the exploration of novel genetic methods for engineering cells at a molecular level (for e.g., with the coupling of CRISPR technology etc) to assessing their targeted utility, such investigative studies have recently led to the FDA announcing, they no longer require animal driven studies [1]. Following biopsy cell isolation and expansion, tissue reconstruction is carried out with a raft of methodologies. These range from basic approaches such as the hanging droplet to more exotic techniques such as lab on chip (exploiting advanced microfluidics), lithography technologies to finally classical and recently discovered contact and non-contact-based jetting/threading approaches.

Although, literature postulates some of these technologies as having capacity to handle and reconstruct tissues, they have not been adopted to clinical use. These result from process limitations to their inability to scale up for the real world. The hanging droplet technique has been around for a long time. However, the technology cannot overcome the process by which it creates tissues to avoid initiating cellular death through necrosis at the core of the generated cell bead. This is largely due to the tightly packed core cells having limited access to oxygen and nutrients [2]. Microfluidics has been developed to mimic 3D disease models. However, the technology is inefficient and limited in the way it creates layered tissues for reasons of scalability [3]. The many manifestations of lithography have also been shown to contribute to this arena. Lithography methodologies have confined themselves to a niche, as the technology can only handle low single cell concentrations, at any given time, and works unfortunately with cacogenic liquids/resins at temperatures which are not conducive to biological activity [4]. These limitations in these three approaches, have highlighted some classical and recently unearthed contact and non-contact technologies as direct cell handling methods, having practicalities which were not available with the three previous approaches. For example., the many manifestations of 3D printing allow cell bearing droplets or filaments to be deposited with relative accuracy in both 2D and 3D. Having said that, these 3D printing technologies have been demonstrated to damage both

* Corresponding author.
E-mail address: s.jayasinghe@ucl.ac.uk (S.N. Jayasinghe).

https://doi.org/10.1016/j.nxmate.2024.100108
Received 8 September 2023; Received in revised form 3 January 2024; Accepted 4 January 2024
Available online 13 February 2024
2949-8228/© 2024 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

biomolecules and cells during the printing process, due to shearing within the needles. These limitations have tempered the hype 3D bio-printing has in its ability to contribute to this enterprise [5].

In the contact and non-contact jetting/threading category, many other technologies have been recently unearthed, and fully validated for demonstrating their ability to handle not only highly concentrated single and multiple cell types, with biomolecules, but also whole fertilised embryos. Post treated cells and embryos have been interrogated with those controls and found to be indistinguishable. The technologies referred to here are bio-electrosprays[6], cell electrospinning [7], aerodynamically assisted bio-jetting/threading [8] to pressure assisted cell spraying and spinning [9]. These approaches operate using different driving mechanisms, but they have one common feature, namely they utilise very large inner bore needles of > 1000 μm and are capable of generating cell bearing residues in a few tens of micrometres. Using large inner bore needles significantly reduces cell shearing within needles, allowing these technologies to process highly concentrated cell suspensions containing multiple cell types with biomolecules etc to fertilised embryos, without causing any damage to them.

That being the case, the authors in this study are focussing on the exploration of aerodynamically assisted bio-jets for processing human adipose-derived stem cells. The post treated cells are evaluated in comparison to controls, at molecular level, for assessing their viability over a 72 hr time course, to finally assessing their capacity to differentiate through well-established cell differentiation assays. These investigative studies place the applicability of aerodynamically assisted bio-jets/threads at par with both bio-electrosprays and cell electrospinning.

2. Experimental set-ups

2.1. Bio-jetting device

Briefly, bio-jetting through aerodynamically assisted bio-jets, takes place, with a needle holding the flow of a cell suspensions, held centrally in a pressurised chamber, with an exit orifice, below it. As the chamber pressure is greater than the atmospheric pressure (surrounding the device), this sees, pressure travelling from high to low. Thus, resulting in a flow field which enables, the liquid flowing within the needle, in this scenario the cell suspension, drawn out of the needle, in the shape of a liquid cone, with its apex and emanating jet protruding through the exit orifice subsequently breaking-up into either a 3D spray plume or a stream of droplets. The jet formation, stability to whether a spray plume or a stream is generated, depends on the liquid properties, liquid flow rate to the needle, distance between the needle exit and exit orifice to finally the applied pressure to the chamber. In some scenarios elevating the applied pressure for a very low flow rate has shown the generation of droplets released from the exit orifice without a protruding jet, this occurs predominantly when the flow rate is very low. The bio-jetting device explored in these studies, was very similar to those devices previously explored by us [10]. The device was manufactured from glass and allowed the needle holding the flow of the cell suspension to be moved both close and away from the exit orifice. The needle had an inner bore diameter of ~1650 μm with a wall thickness of ~800 μm. The exit orifice explored had a diameter of ~700 μm. In these studies, we varied the applied pressure and flow rate from 0–1 bar and 10^{-12}-10^{-9} m^3s^{-1}, respectively. The needle orifice to exit orifice was varied from 0.2–3 mm. For bio-jetting the device was held above a sterile 50 ml falcon tube which was used to collect the jetted cells. Fig. 1 illustrates the workflow explored in these studies. Expanded cells were harvested and spilt into two aliquots namely a) culture controls (sample which was not exposed to any jetting), and b) the sample for bio-jetting.

2.2. Cell culture

Human adipose-derived stem cells (hADSCs) were used in this study. These cells were commercially obtained from the ATCC (ATCC, UK) and cultivated in MesenPRO RS™ basal cell culture medium (ThermoFisher, UK) with 2% MesenPRO RS™ growth supplement (ThermoFisher, UK) and 1% penicillin/streptomycin (Sigma-Aldrich, UK) at 37 °C and 5% CO_2. Cells were passaged routinely with standard protocols, and all experiments were performed at passages 3 or 4.

2.3. Microscopy

Optical microscopy was carried out using a standard inverted Leica DMIL light microscope. Images were taken of both the culture and bio-jetted samples at the same time point over the investigated time course of 72 h (Fig. 2). Imaging for cell differentiation was carried out using an Olympus U-TV0.5XC-3 inverted microscope.

2.4. Assessing cell viability

Flow cytometry was explored for quantifying cellular dynamics over a time course of 72 h. Briefly, the flow cytometry technique can quantify both necrotic (dead cells) and apoptotic (programmed cell death) cells. In those cells undergoing programmed cell death, the membrane phospholipid phosphatidylserine is translocated from the inner to the outer layer of the plasma membrane. During the initial stages of programmed

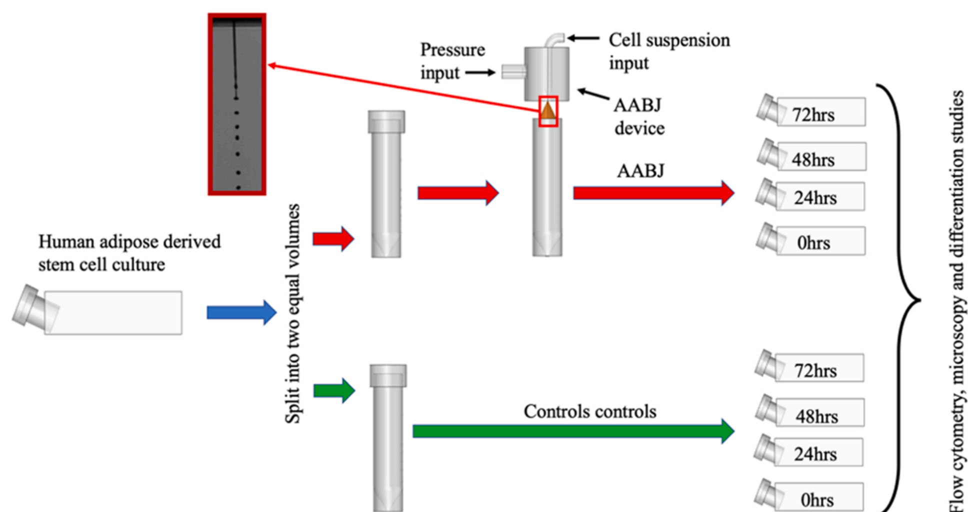

Fig. 1. Illustrates the workflow in these studies. The magnified insert demonstrates bio-jetting the of the cell suspension.

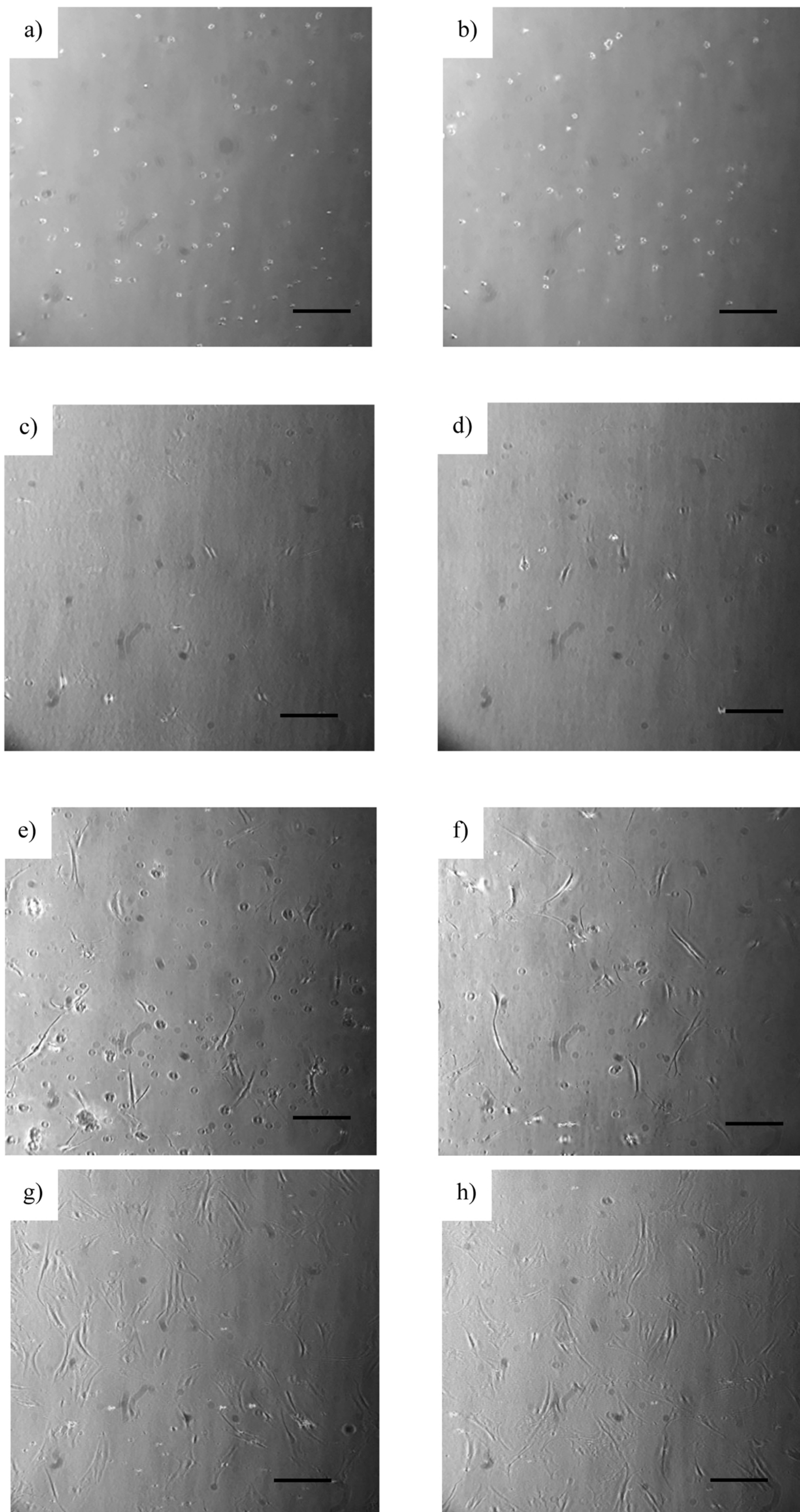

Fig. 2. Representative optical micrographs illustrating both the control (a, c, e, g) and bio-jetted (b, d, f, h) samples, at 0, 24, 48 and 72 h. Scale bar in all panels represent 100 μm.

cell death, the cell membrane remains intact, while at the moment of cell death, the cell membrane loses its integrity and becomes leaky to the dye propidium iodide (PI). Annexin V staining has a high affinity for phosphatidylserine, in combination with PI, allowing the identification of living cells (annexin-, PI-), those undergoing early programmed cells death (annexin+, PI-), dead cells (annexin+, PI+) and finally cellular debris (annexin-, PI+), giving both an accurate and sensitive measurement of the dynamics of the cells health.

Samples of single cell suspensions were prepared from each sample. Cells were mixed with PI (final concentration 1 μg/ml; Sigma, UK) and Annexin V (FITC; final concentration 1 μg/ml; Pharmingen, UK) in the presence of 1.8 mM calcium. Subsequently cells were incubated at room temperature for ~15 min prior to quenching in calcium-containing binding buffer (Pharmingen, UK) and were immediately analysed. A Dako Cytomation CyAn™ ADP flow cytometer was explored in these studies to collect data for 20,000 events. The cytometers excitation was set at 488 nm using an argon laser; while the FITC emission was collected with a 525 ± 20-nm band pass filter, and PI with a 675 ± 20-nm filter. Data was analysed using Summit 4.3 software (Dako Cytomation, UK). In these investigative studies, the controls and bio-jetted cells were passed though the cytometry apparatus to analyse the number of live and dead cells through to cells that were undergoing apoptosis. Flow cytometry analysis was carried out on several cellular samples for both controls and those bio-jetted cells. Cytometry was carried out on both the samples (controls and bio-jetted) at the time points of 0 (as jetted and collected), 24 h, 48 h and finally 72 h (Fig. 3).

2.5. Cell differentiation assays

To study the differentiation ability of the hADSCs, 1×10^5 cells were cultured from each sample (controls and bio-jetted) in a six-well plate and studied for adipogenic, osteogenic and chondrogenic differentiation abilities by using StemPro™ adipogenesis, osteogenesis and chondrogenesis differentiation kit (ThermoFisher, UK) for 14 days. Post time point, these cells were stained for: -

a) *Oil O Red staining (For adipogenesis):* Cells were briefly fixed with 10% neutral buffer saline for 30 min and washed with running water for 5 min. These fixed samples were stained with freshly prepared Oil Red O working solution (0.5 g Oil Red O in 100 ml of isopropanol) for 15 min and rinsed with 60% isopropanol.
b) *Alcian Blue staining (For chondrogenesis): Cells were* fixed in 10% neutral buffer saline for 2 h, brought to stilled water, and stained with 1% Alcian Blue in 3% aqueous acetic acid for 15 min and washed with running water.
c) *Alkaline Phosphatase staining (For osteogenesis):* In this part, the Alkaline Phosphatase detection kit was used (Sigma Aldrich, UK). In brief, cells were fixed in 10% neutral buffer saline for 2 min and rinsed with 1X rinse buffer. Added 0.5 ml Fast Red Violet (FRV) with Naphthol As-Bi phosphate solution and water in a 2:1:1 ratio (FRV: Naphthol: Water) and incubated in the dark at ambient temperature for 15 min. This staining solution was aspirated and rinsed with 1X rinse buffer.

All cell samples were mounted on an Olympus U-TV0.5XC-3 inverted microscope and imaged at 20X (Fig. 4).

The equipment explored in these studies were similar to those explored in our previous investigations. Fig. 1 depicts the spray modes and the equipment arrangement within a laminar flow safety cabinet. Briefly, the bio-jetting device explored in these studies had a chamber volume of approximately 2.4 cm^3, at the top and central to the chamber, was a needle, which accommodated the flow of the cell suspension (supplied via a syringe pump and silicone tube) with an inner bore diameter of ~1650 μm. This needle extended within the chamber, placing its exit approximately 2.5 mm above the chamber exit orifice (based at the bottom of the chamber). The chamber exit orifice had a diameter of ~700 μm. Pressure to the chamber was supplied via a needle having a diameter of ~2000 μm from a side entry to the chamber. Pressure was supplied allowing a pressure range of 0–5 bar with accurate increments of 0.01 bar. Prior to initiating the experiments, all the equipment was sterilised in an autoclave and further cleaned with 70% alcohol. Assessing the operational space/window was carried out with phosphate buffer saline which was supplied to the needle via a syringe pump and silicone tubing. A wide range of applied pressure to flow rate was investigated to assess which applied pressure and flow rate was seen to best generate a stable single steady stream of droplets resulting from a steady jet which underwent varicose break-up. Hence these PBS based studies demonstrated that applied pressure to flow rate of ~1.2 bar to $\sim 10^{-9}$ $m^3 s^{-1}$ was seen to generate a steady stream of droplets from a

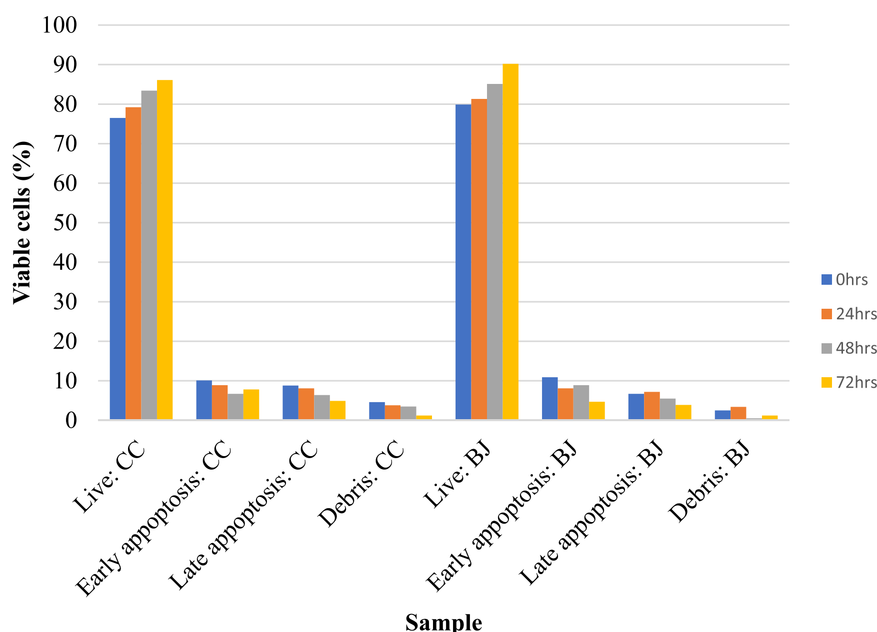

Fig. 3. Cell viability via flow cytometry on culture controls (CC) and those bio-jetted (BJ) cells.

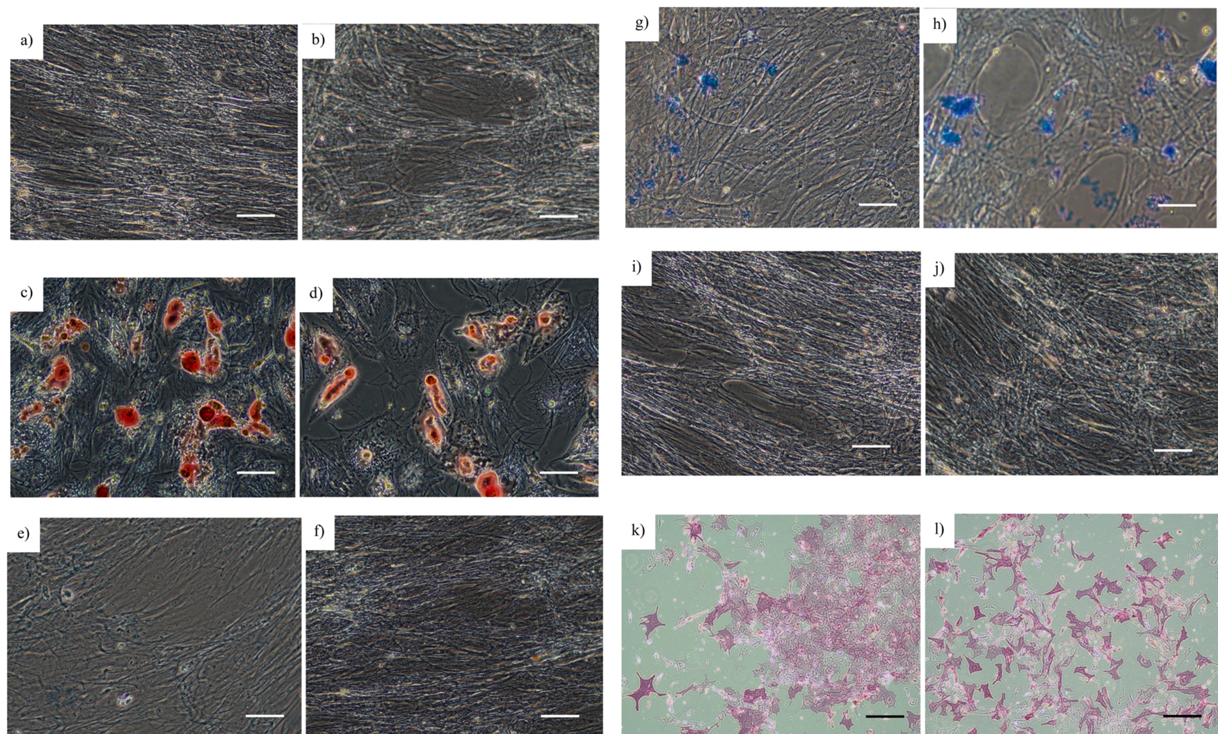

Fig. 4. Charatacteristic inveted microscope images, showing panels a, c, e, g, i and k) culture control samples and, panels b, d, f, h, j and l bio-jetted samples respectively. Scale bar in all panels represent 100 µm. Analysis of staining by light microscopy demonstrated that bio-jetted cells showed no marked difference from control samples.

steady jet and break up process.

With these operational conditions, studies were initiated by exposing several hADSC suspension samples at a concentration of 1×10^6 cells/ml. The bio-jetted cells were directly collected into sterile 50 ml falcon tubes held at ~5 cm below the exit orifice of the bio-jetting device (Fig. 1). On collection these samples were mixed with complete media (MesenPRO RS™ basal cell culture medium with 2% MesenPRO RS™ growth supplement and 1% penicillin/streptomycin). Before these samples where placed in an incubator, the samples were imaged (Fig. 2 panel a) culture control and b) bio-jetted cells). Both bio-jetted and control samples were incubated at 37 °C and 5% CO_2. Panels c) and d) in Fig. 2, depict images of the cells at a time point of 24 h, where the samples were removed from the incubator, imaged and returned to the incubator. Similarly, panels e) and f), depict the samples at the time point of 48 h and finally panels g) and h) represent the time point 72 h. These microscope images demonstrate the samples were indistinguishable. That being said, cells may look comparable but from our previous experiments interrogating cells from a molecular level has been more representative of the cellular dynamics taking place within these cultures. Therefore, as in our previous studies, we exposed these cultures from both controls and bio-jetted samples to flow cytometry.

Prior to using the cytometer, the system was calibrated with fluorescent calibration beads. Aliquots of samples at each given time point (0, 24, 48 and 72 h) for both culture controls and bio-jetted samples were harvested and labelled with molecular probes as per manufacturer protocols and interrogated with a Dako cytometer. Multiple aliquots for each sample for each time point was interrogated and the results demonstrated, control cultures were comparable to those bio-jetted samples. Fig. 3 depicts a bar chart identifying the cellular dynamics for each cell population at the respective time points for the given cell sample. Having established that the bio-jetted and culture control cells are comparable at a molecular level and phenotypically it was essential to established whether these hADSC's retained their capacity to differentiate into the three cell types, characteristic of them, namely, osteogenic, chondrogenic and finally adipogenic.

Similarly, aliquots of both culture controls and bio-jetted cells were cultured in complete media for over 14 days and exposed to a) Oil-o-Red, b) Alcian Blue and finally c) Alkaline Phosphates staining. These stains are markers for hADSC differentiation into the respectively cell lineages, as observed by light microscopy. Fig. 4 depicts representative images of hADSC cultures showing their capacity to differentiate into the respective cell types as identified by the marker stains. Fig. 4, panels a, c, e, g, i and k) represent culture control samples and, panels b, d, f, h, j and l represent the bio-jetted samples respectively.

3. Summary

These investigative studies, interrogating cells jetted via aerodynamically assisted bio-jets have been shown to have no negative effects brought on them by the processing methodology. Similar results were seen on those cells threaded using the sister technology (AABT). Hence this technology provides an alternative approach to both bio-electrosprays and cell electrospinning if the application would require a non-electric field driven direct cell jetting methodology. The technology in our hands will develop into a novel cell printing platform for reconstructing 3D tissues and other cell constructs critical for studying basic cell biology of tissue engineering, and in the wider context of regenerative biology and medicine.

Declaration of Competing Interest

The authors declare no conflicts of interests.

Acknowledgements

SNJ gratefully acknowledges the Royal Society in the United Kingdom, for funding this research in the BioPhysics Group at UCL, through seed corn funding.

References

[1] M. Wadman, FDA no longer needs to require animal tests before human drug trials, Science 379 (2023) 127–128.

[2] S.J. Han, S. Kwon, K.S. Kim, Challenges of applying multicellular tumor spheroids in preclinical phase, Cancer Cell Int 21 (2021) 152, https://doi.org/10.1186/s12935-021-01853-8.

[3] H.F. Chan, S. Ma, K.W. Leong, Can microfluidics address biomanufacturing challenges in drug/gene/cell therapies? *Regenerative*, Biomaterials 3 (2016) 87–98.

[4] H.Q. Xu, J.C. Liu, Z.Y. Zhang, C.X. Xu, A review on cell damage, viability, and functionality during 3D bioprinting, Mil. Med. Res. 9 (2022) 70, https://doi.org/10.1186/s40779-022-00429-5.

[5] (a) G.M. Nishioka, A.A. Markey, C.K. Holloway, Protein damage in drop-on-demand printers, J. Am. Chem. Soc. 126 (2004) 16320–16321;
(b) L. Ning, N. Betancourt, D.J. Schreyer, X. Chen, Characterization of cell damage and proliferative ability during and after bioprinting, ACS Biomater. Sci. Eng. 4 (2018) 3906–3918.

[6] S.N. Jayasinghe, A. Qureshi, P. Eagles, Electrohydrodynamic jet processing: an advanced electric-field-driven jetting phenomenon for processing living cells, Small 2 (2006) 216–219.

[7] A. Townsend-Nicholson, S.N. Jayasinghe, Cell electrospinning: a unique biotechnique for encapsulating living organisms for generating active biological microthreads/scaffolds, Biomacromolecules 7 (2006) 3364–3369.

[8] (a) S. Arumuganathar, S. Irvine, J.R. McEwan, S.N. Jayasinghe, Aerodynamically assisted bio-jets: the development of a novel and direct non-electric field driven methodology for engineering living organisms, Biomed. Mater. 2 (2007) 158–168;
(b) S. Irvine, S. Arumuganathar, J.R. McEwan, S.N. Jayasinghe, Coaxial aerodynamically assisted bio-jets: a versatile paradigm for directly engineering living primary organisms, Eng. Life Sci. 7 (2007) 599–610.

[9] (a) S. Arumuganathar, S. Irvine, J.R. McEwan, S.N. Jayasinghe, A novel direct aerodynamically assisted threading methodology for generating biologically viable microthreads encapsulating living primary cells, J. Appl. Polym. Sci. 107 (2008) 1215–1225;
(b) S.N. Jayasinghe, N. Suter, Pressure driven spinning: a versatile direct approach for preparing nanoscaled functionalised fibres containing structural, functional and/or biological materials, Biomicrofluidics 4 (2010) 014106.

[10] E. Griessinger, S.N. Jayasinghe, D. Bonnet, Aerodynamically assisted bio-jetting hematopoietic stem cells, Analyst 137 (2012) 1329–1333.