Bio-Electrospraying: A Potentially Safe Technique for Delivering Progenitor Cells

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ABSTRACT: Bio-electrospraying is fast becoming an attractive tool for in situ cell delivery into scaffolds for tissue engineering applications, with several cell types been successfully electrosprayed. Bone marrow derived mesenchymal progenitor/stem cells (BMSC), which are an important cell source for tissue engineering, have not been explored in detail and the effect of electrospraying on their “stemness” is not known. This study therefore investigates the effects of electrospraying on BMSC viability, proliferation, and multilineage differentiation potential. Electrospraying a BMSC suspension at flow rate of 6 mL/h and voltages of 7.5–15 kV could successfully generate a continuous, stable and linearly directed electrospray of cells. Morphological observation, trypan blue tests and alamar blue based metabolic assays revealed about 88% of these electrosprayed cells were viable, and proliferated at rates similar to native BMSCs. However, at higher voltages, electrospraying became unstable and reduced cell viability, possibly due to electrical or thermal damage to the cells. BMSCs electrosprayed at 7.5 kV also retained their multipotency and could be successfully differentiated into adipogenic, chondrogenic, and osteogenic lineages, demonstrating similar morphology and gene expression levels as induced native BMSCs. These results indicate that bio-electrospraying could be safely used as a progenitor/stem cell delivery technique for tissue engineering and regenerative medicine applications.

Introduction

Tissue engineering approaches have conventionally relied upon separate sequential techniques for scaffold fabrication and subsequent cell seeding and population of the scaffolds. Such approaches face the limitation of poor cell infiltration and non-uniform cell distribution in three-dimensional (3-D) scaffolds. As the distribution of tissue formed within engineered constructs is related to the initial distribution of cells within the scaffold (Holy et al., 2000), several cell engineering techniques capable of directly delivering living cells in situ into simultaneously fabricated scaffolds have been attempted to overcome this limitation (Jayasinghe et al., 2006a; Yang and El Haj, 2006).

In particular, the use of jet-based techniques to process cell suspensions has been gaining attention due to their ability to directly organize or “print” cells into 3-D architectures to create functional tissue constructs. Electrospraying or electrohydrodynamic jetting is one such jet-based technology wherein a liquid medium is charged to several thousand volts and passed through a large-bore needle to be fragmented into droplets (Eagles et al., 2006). Bio-electrospraying” a suspension of living cells can form micro-droplets of cells without causing any apparent deleterious effect on the cells and can generate finer droplets and process denser cell suspensions than other jetting techniques such as ink-jet printing and aerosol delivery (Jayasinghe et al., 2006a,b). Recent studies have reported successful electrospraying of several mature cell types including smooth muscle cells (Stankus et al., 2006), neuronal cells (Eagles et al., 2006), kidney cells (Kwok et al., 2008), lymphocytes (Kempski et al., 2008), and even multicellular organisms (Clarke and Jayasinghe, 2008; Odenwalder et al., 2007). While more than 70% of the
electrosprayed mature cells have been shown to remain viable in these studies during short-term culture over 1 week (Stankus et al., 2006), few studies have attempted to bioelectrospray stem cells (Mongkoldhumrongkul et al., 2009). Bio-electrosprayed stem cell viability has been demonstrated over a period of 72 h, but a detailed assessment of their fate after extended culture periods, particularly their multilineage differentiation potential, has not been reported yet.

The multilineage differentiation potential of stem cells has been explored for engineering and regeneration of various mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, and adipose tissue (Banfi et al., 2000; Muraglia et al., 2000; Pittenger et al., 1999; Sahoo et al., 2006). The bone marrow is a rich source of hematopoietic stem cells and non-hematopoietic mesenchymal stem/progenitor cells. A subset of these mesenchymal cells that are isolated by preferential adhesion on tissue culture plastic are termed as bone marrow stromal cells (BMSCs) (Prockop, 1997). BMSCs have been frequently used as seed cells in tissue engineering applications due to their ease of isolation, capacity of in vitro self-renewal and expansion, and multipotency.

Bio-electrospraying could be a useful technique to deliver these cells in situ into 3-D scaffolds, so as to improve cell infiltration and distribution in the scaffolds. However, the effects of bio-electrospraying, which involves exposing the cells to high electric fields, on BMSC viability, proliferation and multilineage differentiation potential is yet unknown. For the successful application of electrospraying in stem cell handling, it is essential that the electrosprayed stem cells be interrogated thoroughly to ensure that their viability and bio-functionality are not adversely affected. This study aims to optimize bio-electrospraying of BMSCs and investigate the effect of bio-electrospraying on BMSC viability, proliferation and multipotency.

**Materials and Methods**

**Isolation of BMSCs**

BMSCs were isolated from bone marrow aspirates from five New Zealand White Rabbits (average weight, 2.5 kg) under approval of the NUS Institutional Animal Care and Use Committee, National University of Singapore, according to procedures described previously (Sahoo et al., 2007, 2009a, 2010a,b). Bone marrow aspirates were mixed with culture medium comprising Dulbecco’s modified Eagle’s medium with low glucose (DMEM; Gibco, Invitrogen, Carlsbad, CA) and 15% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% antibiotics (200 units/mL penicillin G and 200 units/mL streptomycin sulfate) and cultured at 37°C in an incubator with 5% humidified CO₂. The cultures were replenished with fresh medium every 3–4 days. BMSCs were isolated by selective adherence to tissue culture polystyrene and non-adherent cells were removed during media change and subculturing. Semi-confluent cells of second passage were used for bio-electrospraying.

**Bio-Electrospraying Set-Up and Optimization**

The bio-electrospraying set-up consisted of a high voltage power supply (RR Series, Gamma High Voltage Research, Ormond Beach, FL), a syringe pump (KD Scientific, Holliston, MA), a retort stand holding an extrusion needle and a laboratory jack holding a cell collecting tissue culture petri dish (Fig. 1). The equipment were sterilized using 70% ethanol and UV irradiation for 30 min within a biosafety cabinet. A suspension of BMSCs with concentrations ranging from 1 × 10⁶ to 6 × 10⁶ cells/mL was delivered via the syringe pump to the extrusion needle that was held at a potential difference with respect to the collector. The resultant electric field caused the cell suspension to be drawn into jets and deposited as droplets on the collecting petri dish placed 15 cm away from the needle. In order to optimize the parameters and obtain stable electrospraying of cells, the applied voltage was varied between 1 and 30 kV and the flow rate of the BMSC suspension varied between 1 and 6 mL/h. The cells collected on the petri dish were evaluated for viability or transferred to multi-well plates for further culture and differentiation studies.

**Viability and Proliferation of Electrosprayed BMSCs**

To evaluate the effect of high voltages on BMSC viability and proliferation, the cell suspension was electrosprayed at voltages of 7.5, 15, and 30 kV and cell viability assessed immediately by trypan blue dye exclusion test.
using non-electrosprayed BMSCs (cell suspension passed through the electrospraying set-up at 0 kV, keeping all other parameters unchanged) as control (n = 4). 0.1 mL of the suspension of electrosprayed BMSCs in DMEM was mixed with an equal volume of 0.4% trypan blue (Gibco) and the number of stained cells as well as the total number of cells counted using a hemocytometer within 5 min.

Electrosprayed BMSCs (3 × 10^5 BMSCs/well) were transferred into separate wells of a 6-well culture plate (NUNC, Roskilde, Denmark) and cultured for 2 weeks. Cell viability and proliferation was monitored using alamar blue dye reduction assay by incubating the scaffolds (n = 3) in complete medium containing 10% alamar blue for 3 h and measuring absorbance values of the media at 570 and 600 nm using a 96-well microplate reader. The percentage reduction in dye, which is proportional to the cell viability in the sample, was estimated following the vendor’s protocol (BioSource International, Camarillo, CA).

**Multilineage Differentiation of Electrosprayed BMSCs**

Based on the stability of the electrospraying process and results from the viability and proliferation tests, a voltage of 7.5 kV and flow rate of 6 mL/h were chosen for electrospraying BMSCs for characterization of their multilineage differentiation potential. Electrosprayed BMSCs were transferred into separate 6-well culture plates (3 × 10^5 BMSCs/well) for osteogenic and adipogenic differentiation and 6 × 10^5 cells were grown in a pellet culture for chondrogenic differentiation. The cultured cells were differentiated into adipogenic, osteogenic, and chondrogenic lineages using established protocols (Indrawattana et al., 2004; Jaiswal et al., 1997; Pittenger et al., 1999; Sahoo et al., 2010a). In brief, adipogenic differentiation was induced by three cycles of induction (using DMEM supplemented with 15% FBS, 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, 10 μg/mL insulin, 0.2 mM indomethacin and antibiotics) and maintenance treatment (using DMEM supplemented with 15% FBS, 10 μg/mL insulin and antibiotics). Differentiation was then detected by Oil Red O staining for neutral lipid droplets found within adipocytes. Osteogenic differentiation of BMSCs was induced under the influence of 10 mM β-glycerophosphate, 0.1 μM dexamethasone, 50 μg/mL t-ascorbic acid 2-phosphate and 10 μg/mL insulin, and Alizarin Red staining was carried out to detect orange-red calcium deposits after 3 weeks. Chondrogenic differentiation was induced by culturing the BMSC pellet in serum-free DMEM with 10 ng/mL TGF-β1, 50 μg/mL t-ascorbic acid 2-phosphate, 1.25 mg/mL BSA, 0.1 μM dexamethasone, 1 × ITS (insulin–transferrin–selenium) and antibiotics supplements. After 3 weeks, the cell pellet was sectioned and stained with alcian blue for cartilage matrix-specific sulfated mucosubstances. All the induction reagents, except ITS (Gibco) and TGF-β1 (R&D Systems, Minneapolis, MN), were from Sigma-Aldrich, St. Louis, MO.

As two separate controls, native BMSCs were either similarly induced into the three lineages (positive controls) or maintained in an undifferentiated condition using expansion medium (negative control).

**Quantitative RT-PCR Analysis of BMSC Differentiation**

In order to confirm BMSC differentiation, gene expression of BMSC markers and several lineage-specific proteins was studied. The gene expression levels in differentiated bio-electrosprayed BMSCs were compared with two separate controls: native BMSCs that were either similarly induced into the three lineages (positive controls) or maintained in an undifferentiated condition using expansion media (negative controls). The BMSC markers used were connective tissue growth factor (CTGF) and matrix gla protein (MGP) (Igarashi et al., 2007). The tissue-specific genes analyzed were fatty acid binding protein-4 (FABP4) and peroxisome proliferator-activated receptor (PPARγ2) for adipogenesis (Chawla et al., 1994; Noer et al., 2007), runt related transcription factor 2 (Runx2), osteopontin (OP) and osteonectin (ON) for osteogenesis (Guillot et al., 2008; Zou et al., 2008), and sox9 and aggrecan (Agg) for chondrogenesis (Indrawattana et al., 2004; Zou et al., 2008). Total RNA was extracted from the induced and non-induced control BMSCs using RNeasy Mini Kit (Qiagen, Valencia, CA), and quantitative reverse transcriptase-mediated PCR (Q-RT-PCR) was performed using SYBR-green chemistry using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene. Primer sequences were designed, using Primer-3 software, from rabbit gene sequences obtained from the NCBI GenBank and RefSeq databases, and synthesized by Research Biobs, Singapore (Table I). cDNA synthesis and PCR expansion (using iScript and iQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA) were performed in a iCycler iQ detection system (Bio-Rad Laboratories). Data were analyzed for relative expression using the ΔΔCt method, and normalized against the basal expression profile of undifferentiated BMSCs (n = 3, for each group).

**Statistical Analysis**

All data were expressed as mean ± standard error. Due to small sample sizes, statistical analyses were performed by non-parametric tests using SPSS Statistics 17.0 statistical software package. Multiple groups (in trypan blue tests and Q RT-PCR results for each gene) were compared by Kruskal–Wallis tests and post hoc Mann–Whitney tests with Bonferroni correction. Multiple group comparisons involving repeated measures (in alamar blue assays) were performed by Friedman’s test and post hoc Wilcoxon tests. P-values less than 0.05 were considered significant.
Results

Bio-Electrospraying Optimization and Cell Viability

Cell suspensions with concentrations of $1 \times 10^6$ and $6 \times 10^6$ cells/mL could be electrosprayed successfully, without requiring any change in electrospraying parameters. The lower concentration of $1 \times 10^6$ cells/mL was chosen to minimize the number of cells required for further studies. At the flow rate of 6 mL/h, cells electrosprayed at low voltages were ejected from the needle in intermittent spurts. As the voltage was gradually increased to 7.5 kV, the output became more stable and was directed linearly and vertically downwards in the form of a narrow near-continuous jet towards the collector depositing cells at the rate of $1 \times 10^5$ cells/min. At voltages beyond 15 kV, the electrospray became highly divergent and unstable, with only a few droplets being directed to the collector, making the conditions unsuitable for this particular study. A sterile polystyrene tube was placed 5 cm below the jetting needle to enable collection of all the electrosprayed cells into the collecting petri dish (for assessment of cell viability and proliferation).

The viability of BMSCs after electrospraying at different voltages (0 kV: 91.5 ± 1.2%, 7.5 kV: 88.7 ± 1.2%, 15 kV: 87.8 ± 1.1%, 30 kV: 83.1 ± 0.7%) were significantly different (Kruskal–Wallis, $P = 0.011$), with BMSCs electrosprayed at 30 kV being significantly less viable compared to the other groups (post hoc Mann–Whitney U-tests, $P = 0.029$) (Fig. 2).

Proliferative Capacity of Electrosprayed BMSCs

Phase contrast microscopy showed that the 7.5 and 15 kV electrosprayed and control BMSCs attached to the tissue culture polystyrene (TCP) substrate within 1 h of incubation and began to develop an elongated morphology within 3 h, becoming confluent at 7 days of culture. In contrast, BMSCs electrosprayed at 30 kV failed to attach even after 24 h of culture (Fig. 3), further indicating their loss of viability.

Alamar blue dye reduction assay showed a gradual and significant increase in metabolic/proliferation rate of control BMSCs (0 kV) as well as the BMSCs electrosprayed at 7.5 and 15 kV, during the 2 weeks of culture (Friedman, $P = 0.028$); Alamar blue % reduction by the BMSCs electrosprayed at 7.5 and 15 kV increased from 40.7 ±

### Table 1. Primer sequences designed for real-time RT-PCR assays.

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Figure 2. Trypan blue dye exclusion assays ($n = 4$) showed that BMSCs remained viable after electrospraying (ES). Increasing electrospraying voltage was associated with a decreasing trend in viability, with the 30 kV ES BMSCs showed significantly lower viability compared to the other groups ($P < 0.05$).
3.9% to 82.1 ± 3.5% and from 38.8 ± 0.9% to 76.9 ± 0.4%, respectively, while that of the control increased from 49.5 ± 4.0% to 87.1 ± 1.2%, between day 3 and day 14 (Fig. 4). The values for 30 kV electrosprayed cells remained constant throughout the culture period at about 33% (Friedman, $P = 0.361$); while microscopy revealed that there were no viable cells in this group after 24 h (Fig. 3), the calculated 33% reduction value is likely a measurement artifact possibly due to the broad and overlapping absorption spectra of oxidized and reduced alamar blue and the contribution of serum proteins in the culture media, which are not fully corrected by the mathematical formula used to estimate dye reduction (Goegan et al., 1995).

Increasing the electrospraying voltage was observed to reduce cell viability with significant differences noted between the viability of cultured BMSCs electrosprayed at different voltages (Kruskal–Wallis; day 3: $P = 0.012$, day 7: $P = 0.006$, and day 14: $P = 0.001$) (Fig. 4). While the 30 kV group had significantly lower readings compared to the other groups, the 15 kV electrosprayed BMSCs had significantly lower viability compared to the 0 kV control group at all time points (Mann–Whitney, $P = 0.049$). At the end of 2 weeks, 7.5 kV electrosprayed BMSCs had significantly higher viability compared to the 15 kV group.
(Mann–Whitney, $P = 0.049$), with no significant difference compared to the 0 kV controls (Mann–Whitney, $P = 0.275$).

**Multilineage Differentiation of Electrosprayed BMSCs**

After adipogenic induction, Oil Red O staining showed cytoplasmic lipid droplets within several induced-electrosprayed and induced-native BMSCs (indicated by arrows in Fig. 5), but not within the non-induced BMSCs. Cytoplasmic lipid droplets started appearing at the end of the 1st cycle of induction and gradually increased in number with each cycle. Alizarin Red S staining of osteogenic induced cultures showed orange-red deposits of calcium in the induced-electrosprayed and induced-native BMSCs, whereas no calcium deposits were found in the non-induced BMSC cultures. Similarly, alcian blue staining after chondrogenic induction revealed abundant blue-stained mucosubstances, characteristic of chondrocytes, in the induced cultures of electrosprayed and native BMSCs, while the non-induced cultures showed only a faint blue staining (Fig. 5).

Quantitative RT-PCR analysis after multilineage differentiation of BMSCs corroborated the histological evidence. A significant upregulation of most lineage-specific markers (with the exception of FABP4 and OP) and a significant downregulation of the BMSC markers were observed in the induced electrosprayed BMSCs and the induced native BMSCs (positive controls), compared to the non-induced BMSCs (negative controls) (Kruskal–Wallis, $P < 0.05$). The BMSC markers, CTGF and MGP, were either not expressed or expressed at very low levels in the induced cultures. There were no significant differences in expression levels of the lineage-specific genes (with the exception of aggrecan) in the induced-electrosprayed BMSCs and the positive controls (post hoc Mann–Whitney tests, $P > 0.05$). Data with statistically significant differences (significant Kruskal–Wallis and post hoc Mann–Whitney) are indicated in Figure 6.

**Discussion**

The study showed that electrospraying of BMSCs at 7.5–15 kV could successfully produce a stable electrospray of cells that retained the viability, proliferative ability, and multipotent characteristics of native BMSCs. While the electrosprayed jet at 7.5 kV was continuous, stable and directed vertically downwards over a small area on collecting petri dish, electrospraying at voltages over 15 kV destabilized...
the electrospray, causing discontinuous, haphazardly directed jetting, resulting in increased cell loss. Such diffuse and chaotic spraying would prevent the applicability of the technique for cell delivery applications. Similar instability at high voltages has been previously reported, where it has been ascribed to high electrical conductivity and low viscosity of the electrosprayed cell suspension used ($1 \times 10^6$ cells/mL) (Mongkoldhumrongkul et al., 2009). While the current study did not systematically study the effect of viscosity, cell suspensions of $1 \times 10^6$ and $6 \times 10^6$ cells/mL could be stably electrosprayed, without requiring any change in electrospraying parameters. This suggests that high cell concentrations used in this study could have resulted in suspensions exceeding a critical viscosity to influence electrospray stability. However, viscosity effects of low cell concentrations could also be eliminated by altering the cell spraying medium through inclusion of synthetic and natural hydrogels in the cell suspension (Stankus et al., 2006; van Aalst et al., 2008).

Cellular viability, as observed in the trypan blue dye exclusion assays, indicated that cell membrane integrity was preserved in the BMSCs electrosprayed at 7.5–15 kV as in non-electrosprayed controls. Electrospraying at a higher voltage of 30 kV was not only unstable, but was also associated with a significant loss of viability, as observed by the trypan blue assay, immediately after electrospraying. While the possible effect of the modified collecting mechanism (sterile polystyrene tube placed between the jetting needle and collecting petri dish) used at 30 kV on the cell viability was not studied, it is known that strong external electric fields during electrospraying (0.5–2.0 kV/cm) can potentially induce pore formation and cell membrane damage similar to electroporation, leading to loss of cell homeostasis and cell death (Davalos and Rubinsky, 2008; Gass and Chernomordik, 1990; Ho and Mittal, 1996; Tsong, 1991). Membrane damage manifesting as extrusions and blebs, which are frequently seen in electroporated cells (Gass and Chernomordik, 1990; Lu et al., 2005), were observed in the 30 kV electrosprayed cells in this study. In addition to electrical damages to the cell membrane, which can be reversible or irreversible depending on the electric field strength and cell susceptibility (DeBruin and Krassowska, 1999), high voltages can also induce thermal damage in the cells (Chen et al., 1998), contributing to their decreased viability and proliferation. Although trypan blue assay suggested that 83% of cells were viable after electrospraying at 30 kV, most of these cells failed to attach and proliferate on the TCP substrate. This may be explained by the possibility of sub-lethal or intracellular damages to cells that manifested as poor cell viability on continued culture. Overestimation of cell viability by the trypan blue dye exclusion assay could also be a contributing factor. Trypan blue assays estimate cell viability relying on exclusion of the dye by healthy cell membranes and its selective intracellular accumulation through the permeable membranes of dead cells; these assays fail to identify not only dying and apoptotic cells, but also cells with intra-cytoplasmic damage.

Figure 6. Quantitative RT-PCR analysis ($n = 3$) corroborated the histological evidence that the electrosprayed BMSCs possessed adipogenic, osteogenic, and chondrogenic differentiation potential. A significant upregulation of lineage-specific markers and downregulation of BMSC markers were observed in the induced electrosprayed and native BMSCs (positive controls), compared to the non-induced BMSCs (negative controls) ($P < 0.05$ based on Kruskal–Wallis and post hoc Mann–Whitney tests).
such as that incurred by thermal injury, and generally overestimate viability in such cell populations (Altman et al., 1993; Mascotti et al., 2000).

Since electrospraying at 7.5 kV was stable and did not negatively affect cell viability and proliferation, 7.5 kV electrosprayed cells were chosen for further characterization of BMSCs for demonstration of their multipotency. Histochemical staining as well as gene expression analysis (upregulation of lineage-specific markers and downregulation of BMSC markers) showed that electrosprayed BMSCs could be successfully differentiated into adipogenic, chondrogenic, and osteogenic lineages. No significant differences could be observed in expression levels of most lineage-specific genes after induction of electrosprayed and control BMSCs, indicating that the multipotency of the progenitor cells was preserved after electrospraying. It was observed that while induced native BMSCs stopped expressing the BMSC markers, the induced bio-electrosprayed BMSCs still expressed them at low levels; in addition, bio-electrosprayed BMSCs after chondrogenic induction expressed aggrecan at a significantly lower level than the induced native BMSCs. This suggests the possibility that some of the bio-electrosprayed BMSCs might have retained their BMSC phenotype instead of differentiating along terminal lineages. Further investigation using human mesenchymal stem cells (MSCs; as compared to the heterogeneous population of primary rabbit BMSCs used in this study), including additional differentiation markers and larger sample sizes (to allow parametric data analysis) would be required to obtain a better understanding of the stemness of bio-electrosprayed stem cells.

A recent study has reported the successful electrospraying of immortalized human MSCs: a viability of more than 98% (based on propidium iodide staining and flow cytometry) without any apoptotic cell death was reported, and the cells proliferated at rates similar to control cells over 3 days (Mongkoldhumrongkul et al., 2009). While the observed difference in cell viability (98%, compared to 88% observed in this study) could be due several factors such as differences in the electrospraying conditions, varying cell susceptibility to damages during electrospraying, as well as different sensitivity of the viability test used, both studies indicate the safety and potential of use of electrospraying technique for progenitor/stem cell delivery. It was observed in this study) could be due several factors such as differences in the electrospraying conditions, varying cell susceptibility to damages during electrospraying, as well as different sensitivity of the viability test used, both studies indicate the safety and potential of use of electrospraying technique for tissue engineering. When combined with the technology of electrospinning, bio-electrospraying has the potential to create nanofibrous scaffolds with a uniform 3-D distribution of multipotent BMSCs for use in various tissue engineering and regenerative medicine applications.

**Conclusion**

This study shows that electrospraying of BMSCs at 7.5–15 kV could successfully generate a stable, linearly directed electrospray of progenitor cells. The preserved viability, proliferative ability, and multilineage differentiation potential of such electrosprayed BMSCs indicate that bio-electrospraying could be safely used as a progenitor/stem cell delivery technique for tissue engineering. When combined with the technology of electrospinning, bio-electrospraying has the potential to create nanofibrous scaffolds with a uniform 3-D distribution of multipotent BMSCs for use in various tissue engineering and regenerative medicine applications.

**References**


