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Carbon Nanotubes and Mesenchymal Stem Cells: Biocompatibility, Proliferation and Differentiation

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ABSTRACT

The synergy of the unique properties of carbon nanotubes (CNT) with the remarkable potential of human mesenchymal stem cells (hMSC) provides an exciting opportunity for novel therapeutic modalities. However, little is known about the impact of CNT on hMSC behavior. We report the effect of CNT on hMSC renewal, metabolic activity, and differentiation. Furthermore, we tracked the intracellular movement of CNT through the cytoplasm to a nuclear location and assessed effects on cellular ultra structure.

Over the past two decades there have been significant advances in stem cell therapy and tissue engineering for the repair and replacement of damaged tissues and organs. In parallel, nanotechnology has emerged showing great potential for the creation of the next generation of materials. In particular, carbon nanotubes (CNT) have generated great excitement because of their remarkable optical, mechanical, and electrical properties.[1−6] CNT are helical structures, approximately 1−30 nm in diameter with lengths greater than 100 nm.[7] Depending on their chirality they can be either magnetic or electrically conductive. Since their discovery by Iijima in 1991,[8] a range of CNT have been refined including single wall nanotubes (SWNT), multiwalled nanotubes (MWNT), and functionalized nanotubes. More recently, as illustrated in the comprehensive review by Harrison, CNT have been proposed for biomedical applications such as cell tracking and labeling, tissue engineering scaffolds, nanosensors, and vehicles for controlled release of drugs or delivery of bioactive agents.[9]

Previous research has shown that SWNT have no adverse effect on macrophages, and more importantly, the fluorescence properties of the CNT can be exploited to observe and image the cells. In a recent study by Leeuw,[10] near-IR nanotube fluorescence was imaged from intact living Drosophila larva, where it was revealed that the viability and growth were not adversely affected by SWNT ingestion. Furthermore, a recent report by Chin revealed that CNT coated with a design peptide Nano-1 were taken up by HeLa cells[11] presenting an exciting opportunity for CNT in stem cell therapy, where CNT can be used for cell tracking or labeling or act as a carrier for small particles. However, very little is known about the effect of CNT on stem cell response. Therefore the main aim of this study is to examine the effect of CNT on human mesenchymal stem cell (hMSC) viability, proliferation, and phenotype.

Initially, a range of different types of CNT were screened, including SWNT, MWNT, COOH-functionalized SWNT, and OH-functionalized MWNT, to identify the optimum type of CNT for use with hMSC. To this end, 1.6 mg of high quality CNT (Nanocyl, Belgium) was added to 50 mL hMSC media (DMEM-low glucose containing 10% selected fetal bovine serum and 1% antibiotic) and agitated for 24 h using a magnetic stirrer bar. Subsequently, the supernatant was removed, and the dispersion of CNT in the media was assessed using three methods, that is, visually, using absorption spectroscopy[12] (UV-1601 spectrophotometer, Shimadzu U.K.), and using conductance measurements.[13] The conductance of the media solutions containing the CNT was determined using a standard resistance meter, consisting of two platinum probes (UT70B, Unison, U.K.). Resistance values were measured by the probes over a 72 h period, and the data was continuously collected using a data acquisition card connected to a computer. These values were subsequently used to determine the conductance values (conductance = 1/resistance). From the color of the solutions, it could be seen that both functionalized carbon nanotube systems produced the greatest degree of dispersion. This is echoed

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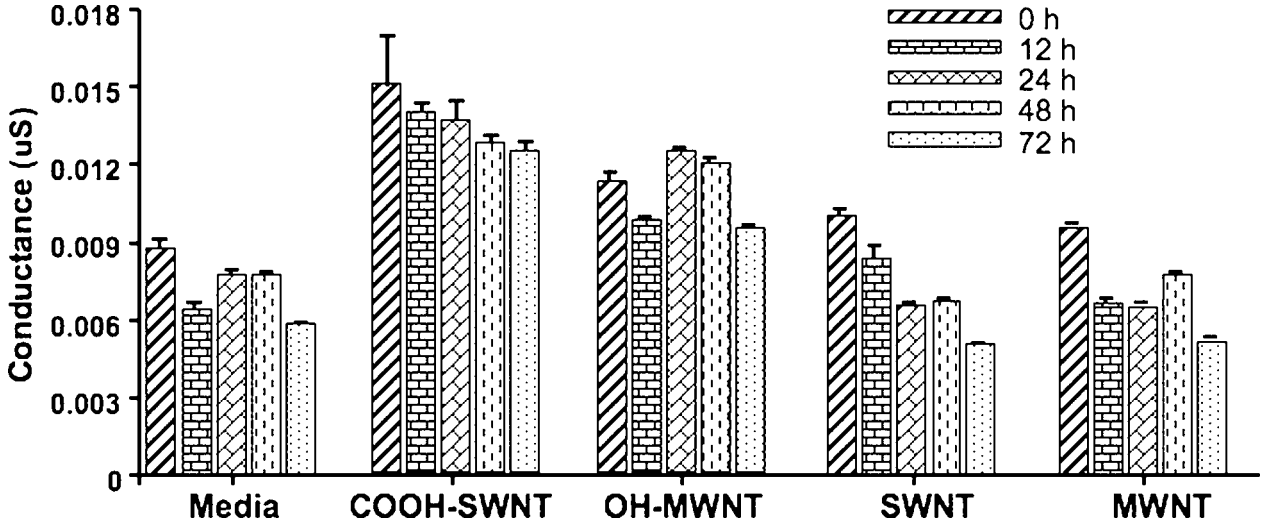

Figure 1. Conductance values of the hMSC media, COOH functionalized SWNT, OH-functionalized MWNT, SWNT, and MWNT in hMSC media over a 72 h time period. Error bars represent standard deviation of the mean ($n = 4$).

in the absorbance spectroscopy (data not shown) and the conductance results, where the highest values of 0.015 μS and 0.012 μS were recorded after 24 h for the COOH-functionalized SWNT and the OH-functionalized MWNT solutions, respectively (Figure 1A).

Since concern still exists about the cytotoxic effects of CNT, the effect of CNT concentration on hMSC viability was examined using a methodology previously developed for optimizing drug concentrations.[14] The hMSC were isolated from adult bone marrow as described previously by Murphy.[15] In brief, a 30 mL aspirate of bone marrow was obtained from the iliac crest of healthy volunteers after informed consent and approval by the Clinical Research Ethical Committee at University College Hospital, Galway. The aspirate was heparin treated and washed in Dulbecco's phosphate-buffered saline solution (D-PBS). Cells were recovered by centrifugation and washed with serum free hMSC media. The red blood cells were lysed in 4% acetic acid, and the mononuclear cells (MNCs) were counted using a hemocytometer. MNC were seeded at a density of approximately 237 000−339 000 cells/cm^2 in hMSC media. After approximately 14 days in primary culture, adherent colonies of hMSC were detached by treatment with 0.25% trypsin and 0.53 mM EDTA and subsequent passages seeded at 5.7×10^3 cells/cm^2. The stem cell phenotype of the hMSC preparations was confirmed by positive differentiation into adipocytes, osteocytes, and chondrocytes.

Subsequently, 3000 hMSC/cm^2 were seeded per well of a 6-well plate. After 24 h, the cells were exposed to aseptically prepared CNT suspensions of 0.00128, 0.0064, 0.032, 0.16, and 0.8 mg/ml COOH-functionalized SWNT or OH-functionalized MWNT. An AlamarBlue™ assay was employed to measure the metabolic activity of the mitochondria of the cells using a colorimetric assay. Briefly, the cells were cultured in a monolayer for 24 h and fed with media containing the CNT for a further 24 h. Thereafter, the media was changed every 48 h. After 6 days, the media was removed, and the cells are washed twice with PBS. The dye was added to the cells, which resulted in a color change of the solution from blue to pink. The supernatant was removed and centrifuged at 10 000 g for 5 min, to ensure there are no free CNT present before measuring the absorbance (530 nm excitation/590 nm emission) on a microplate reader (FLX800, Biotek Instruments, Inc.). Furthermore, the total number of viable cells was determined using a Guava Cytosoft (Guava Technologies, Philadelphia, PA) cell sorter after staining for unviable and total cells using the Guava Viacount reagent. In brief, hMSC and hMSC exposed to CNT were trypsinized and resuspended in the fluorescent ViaCount mixture, and the number of viable and unviable cells were counted using the sorter. Debris, including any CNT that may be present in the cell preparation after extensive washing, was excluded from results by gating based on negative staining with the nuclear dye and particle size. At higher concentrations of both COOH-functionalized MWNT (Figure 2A) and the OH-functionalized SWNT (Figure 2B), CNT appear to have a detrimental effect on the cells. Cell morphology is altered (Figure 2A and B), and cell proliferation (Figure 2C) and cell metabolic rate are reduced (Figure 2D). However, at the lowest concentration of COOH-functionalized SWNT used (0.00128 mg/ml), cell metabolic rate appears to be enhanced compared with control. Furthermore, 0.032 mg/ml of COOH-functionalized SWNT was the highest concentration of CNT that was least toxic to the cell. (Figure 2C,D). At lower CNT concentrations, the viability of cells remaining on the plate at 6 days was greater than 88%. However, with CNT concentration greater than 0.032 mg/ml, not only was the total number of viable cells significantly reduced (Figure 2C), but also the viability of adherent cells was decreased to 81% after exposure to 0.8 mg/ml CNT.

To examine the effect of 0.032 mg/ml of COOH-functionalized SWNT on hMSC proliferation, a growth curve was created. Cumulative doubling was assessed over an 18 day period for two donors and compared to hMSC grown in the absence of CNT. The hMSC were seeded in 6 well plates

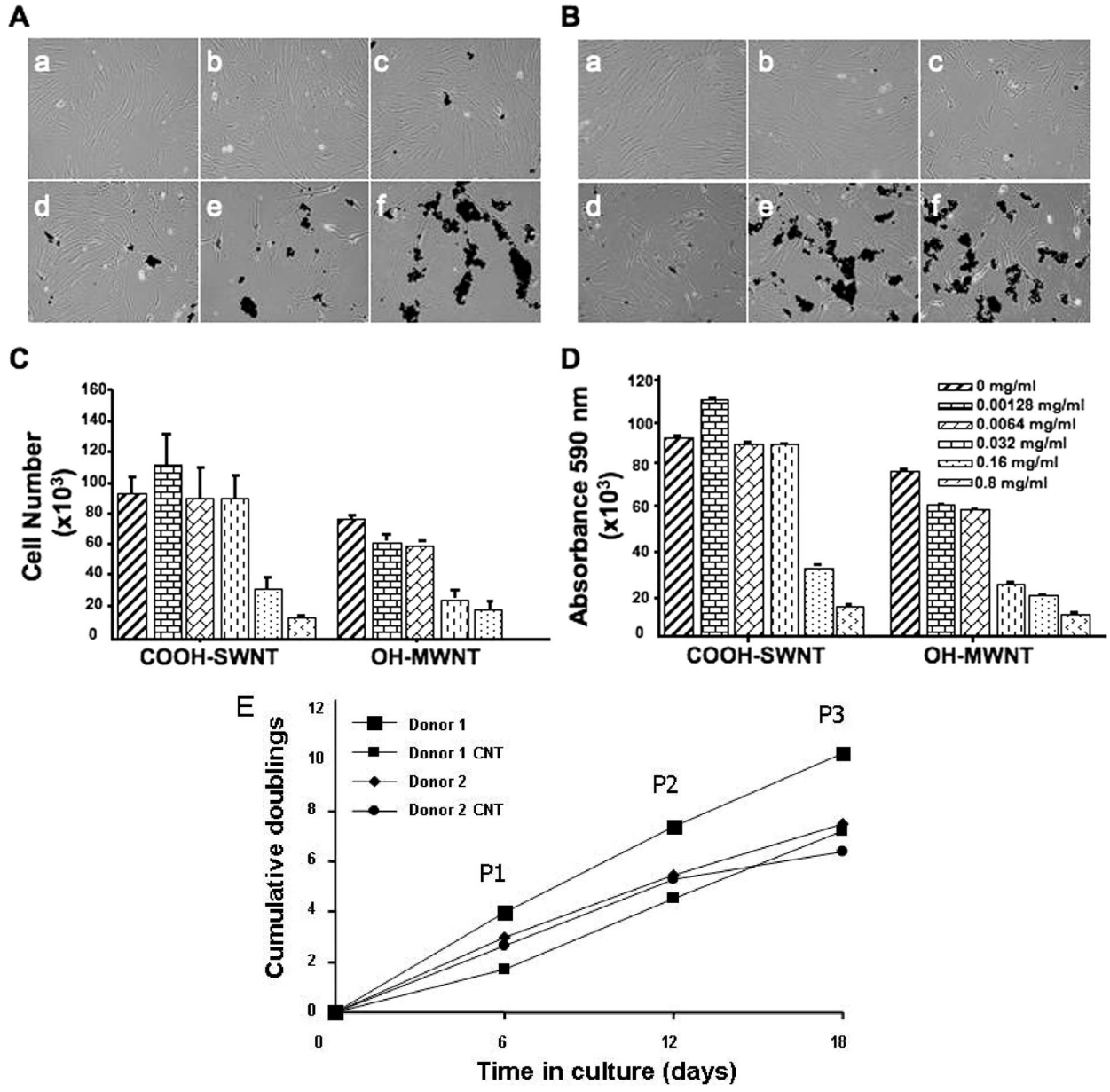

Figure 2. Cell viability in the presence of (A) COOH-functionalized SWNT and (B) OH-functionalized MWNT, (C) viable cell number, (D) cell metabolic activity. a−f represent the various concentrations of nanotubes from 0 mg/ml to 0.8 mg/ml, respectively. Error bars represent standard deviation of the mean ($n = 3$). (E) Cell proliferation for 2 donors from passage 1 to the end of passage 3 after exposure to 0.032 mg/ml COOH functionalized SWNT.

at 3000 hMSC/cm^2 and exposed to 0.032 mg/ml of COOH-functionalized SWNT after 24 h. Thereafter, hMSC were grown for 6 days as shown in Figure 2E. It appears that, in the case of donor 1, exposure to CNT resulted in an initial decrease in proliferation. However, in subsequent passages, the growth rate for treated and untreated cells was equivalent. For the second donor, there appears to be no difference at earlier time points.

To determine whether CNT had any effect on media components, various concentrations of COOH-functionalized SWNT were dispersed in media for 24 h; the suspension was centrifuged, and the supernatant was used as growth media for hMSCs for 6 days (Figure 3). Increasing concentrations of COOH-functionalized SWNT significantly impacted cell proliferation suggesting that the CNT were binding to nutrients in the media required for cell growth. However, the viability of adherent cells exposed to CNT-depleted media was high and equivalent to control cells (results not shown).

Using microscopy, we were unable to definitively determine whether the effects of CNT noted above were a result of uptake or absorption to the cell surface. Previous studies on HeLa cells indicated that cellular internalization of SWNT occurred without the need for external transporter systems[16,17] and the endocytotic mechanism involved was independent of the CNT-conjugation scheme used. To establish if the CNT migrated through the cell wall into hMSCs, a CNT tracking study was conducted by labeling the CNT. To this

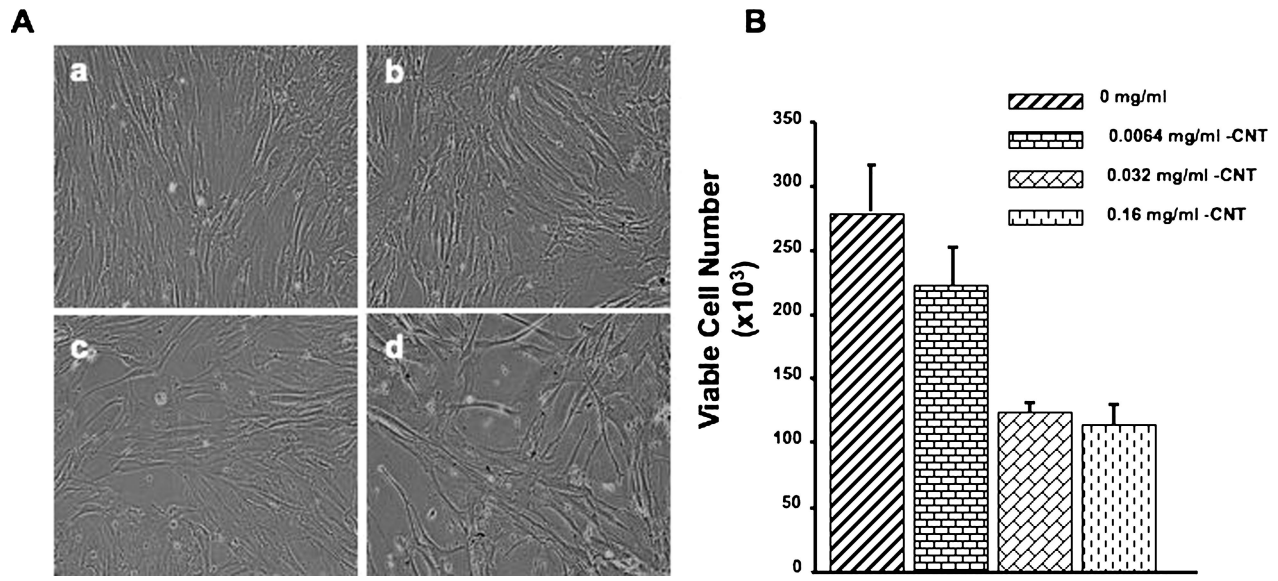

Figure 3. Effects of COOH-functionalized SWNT on media. Varying concentrations of CNT were dispersed in media for 24 h; the CNT were displaced by centrifugation and hMSC fed with the CNT free supernatant for 6 d. (A) hMSC exposed to (a) normal media (b) 0.0064 mg/m, (c) 0.032 mg/ml, and (d) 0.16 mg/ml CNT media (mag. 10×). (B) Cell viability after 6 days in culture. Error bars represent standard deviation of the mean ($n = 4$).

end, COOH-functionalized SWNT were biotinylated with 1×10^{-3} mol NHS biotin (Soltec Ventures, USA) using a method previously described by Sainsbury;[18] the cells were then exposed to 0.032 mg/ml of the biotinylated CNT for 24 h. Biotinylated CNT were detected using streptavidin-FITC (BD Biosciences); the actin cytoskeleton was visualized using phalloidin rhodamine and using DAPI as a nuclear counter-stain. The detection of FITC in the green channel showed that biotinylated COOH-functionalized SWNT were taken up by the hMSC and primarily occupied a cytoplasmic location for the first 24 h. After 6 days in culture, a significant number of the labeled nanotubes had assumed a nuclear location (Figure 4Ac). The hMSC that were not exposed to CNT did not show any evidence of green fluorescence (Figure 4Ad).

Transmission electron microscopy (TEM) was subsequently performed to investigate if the CNT had an effect on cellular ultra structure; hMSC were exposed to 0.032 mg/ml COOH-functionalized SWNT until confluent and then seeded on alginate.[19] After 24 h, the alginate−hMSC constructs were fixed in 3% gluteraldehyde, dehydrated, and sectioned at −20 °C. From the images taken, the organelle structures were preserved when exposed to CNT (Figure 4B).

Subsequently, the effect of CNT on hMSC phenotype, that is, the ability of stem cells to differentiate to multiple lineages, was examined. In particular, adipogenesis, osteogenesis, and chondrogenesis assays were conducted in the presence of CNT. Initially, 3000 hMSC/cm^2 were seeded on T175 tissue culture flasks and exposed to 0.032 mg/ml of COOH-functionalized SWNT for 24 h. Cells were maintained in culture until confluent and were passaged using 0.25% trypsin-EDTA. For differentiation to the adipogenic phenotype, 200 000 cells/cm^2 were seeded onto 6 well plates, and the cells were exposed to 3 rounds of adipogenic induction media containing high glucose Dulbecco's modified essential medium (HG-DMEM), 10% serum, dexamethasone, indomethacin, 3-isobutyl-1-methyl-xanthine, and insulin for 3 days, with a resting period of 1 day in adipogenic maintenance media containing HG-DMEM and insulin. Differentiated hMSC were stained with oil red O to identify lipid-containing vacuoles indicative of positive adipogenesis. Furthermore, the oil red O was extracted using isopropanol, and absorbance was measured at a wavelength of 590 nm (Wallac Victor^3 1420 multilabel counter, Dublin). As shown in Figure 5A,B, hMSC stained positive for adipocytes in the presence of the COOH-functionalized SWNT, and there appears to be no significant difference in the absorbance values recorded for hMSC with or without CNT.

In the case of the osteogenesis, 3000 hMSC/cm^2 were seeded onto 6 well plates. After 24 h, the SWNT-exposed cells were treated with 10% serum media containing osteogenic supplements (dexamethasone, ascorbic acid, and β glycerophosphate) for 16 days, with the media changed every third day. Von Kossa staining and a calcium assay determined the end point of this assay. As for adipogeneis, the presence of CNT had no effect on the amount of calcium deposited by the hMSC that differentiated to the osteogenic phenotype as seen qualitatively using Von Kossa staining (Figure 5C) and quantitatively by the calcium assay results (Figure 5D).

The capacity of hMSC to undergo chondrogenesis was assessed in an aggregate assay where 200 000 hMSC/pellet were centrifuged and exposed to chondrogenic supplements; ITS, HG-DMEM, L-proline, dexamethasone, ascorbic acid, sodium pyruvate, and TGF-β3 for 21 days. Chondrogenesis was determined by measuring the amount of sulfated glycosaminoglycan produced per unit of DNA and also by staining pellets with toluidine blue[20] (Figure 5E,F). In contrast to previous studies where histological methods were used

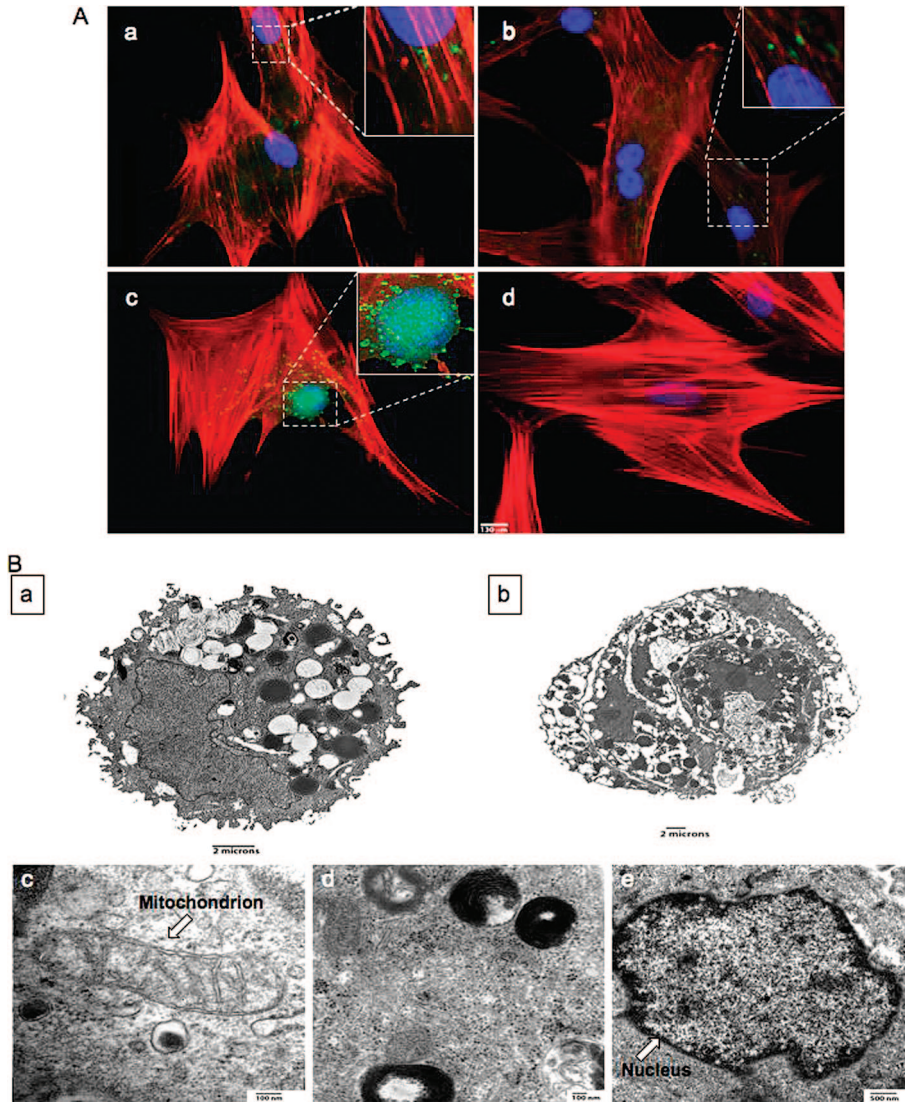

Figure 4. (A) Uptake of COOH-functionalized SWNT by the cell. Fluorescent images of biotinylated CNT within the cell after (a) 24 h, (b) 48 h, and (c) 6 days and (d) hMSC alone (scale bar 130 μm). (B) Transmission electron microscopy images of (a) hMSC seeded on alginate and (b) hMSC exposed to COOH functionalized SWNT and seeded on alginate for 24 h, (c) mitochondria, (d) cytoplasm and ribosomes on the endoplasmic reticulum, (e) and cell nucleus.

to examine the chondrogenic potential of feridex-labeled hMSC,[21] the images shown in Figure 5E revealed that the CNT treated MSC differentiated into chondrocytes. However, in comparison to the adipogenesis and osteoegensis assays, there was a slight decrease in chondrogenesis.

In this study, the effect of the presence of CNT on hMSC viability, proliferation, and differentiation was examined. Initially, it has been revealed that the functionalized CNT were easier to disperse in hMSC media and that the COOH-functionalized SWNT were least toxic to the cells, which correlated with the study conducted by Chin,[11] where it was also noted that SWNT did not adversely alter HeLa cell viability. Although, CNT appeared to sequester nutrients from the media, this did not result in increased levels of cell death, as was the case after direct exposure to high levels of CNT. By using a fluorescent label for tracking, SWNT were shown to migrate through the cell wall to a nuclear location after 24 h. TEM revealed no change to the cellular ultrastructure, correlating with findings from Kam[17,18] and Yehia,[22] where uptake of SWNT did not adversely affect HeLa cells at equivalent CNT concentration. Moreover, the COOH-functionalized SWNT had no significant effect on adipogenesis, osteogenesis, or chondrogenesis. This is the first study to examine the effect of CNT on hMSC and as such is important for new and emerging technologies in drug delivery, tissue engineering, and regenerative medicine.

At present, the authors are developing a novel electrophysiological environment for electrically stimulating hMSC to promote differentiation toward a cardiomyocyte lineage. Of particular interest are CNT based scaffolds with degradable polymer matrices for the generation of functional electroactive patches for damaged cardiac muscle. Therefore, the fact that the CNT had no adverse effects on cell biocompatibility, proliferation, or differentiation augers well for future approaches to tissue repair/regeneration.

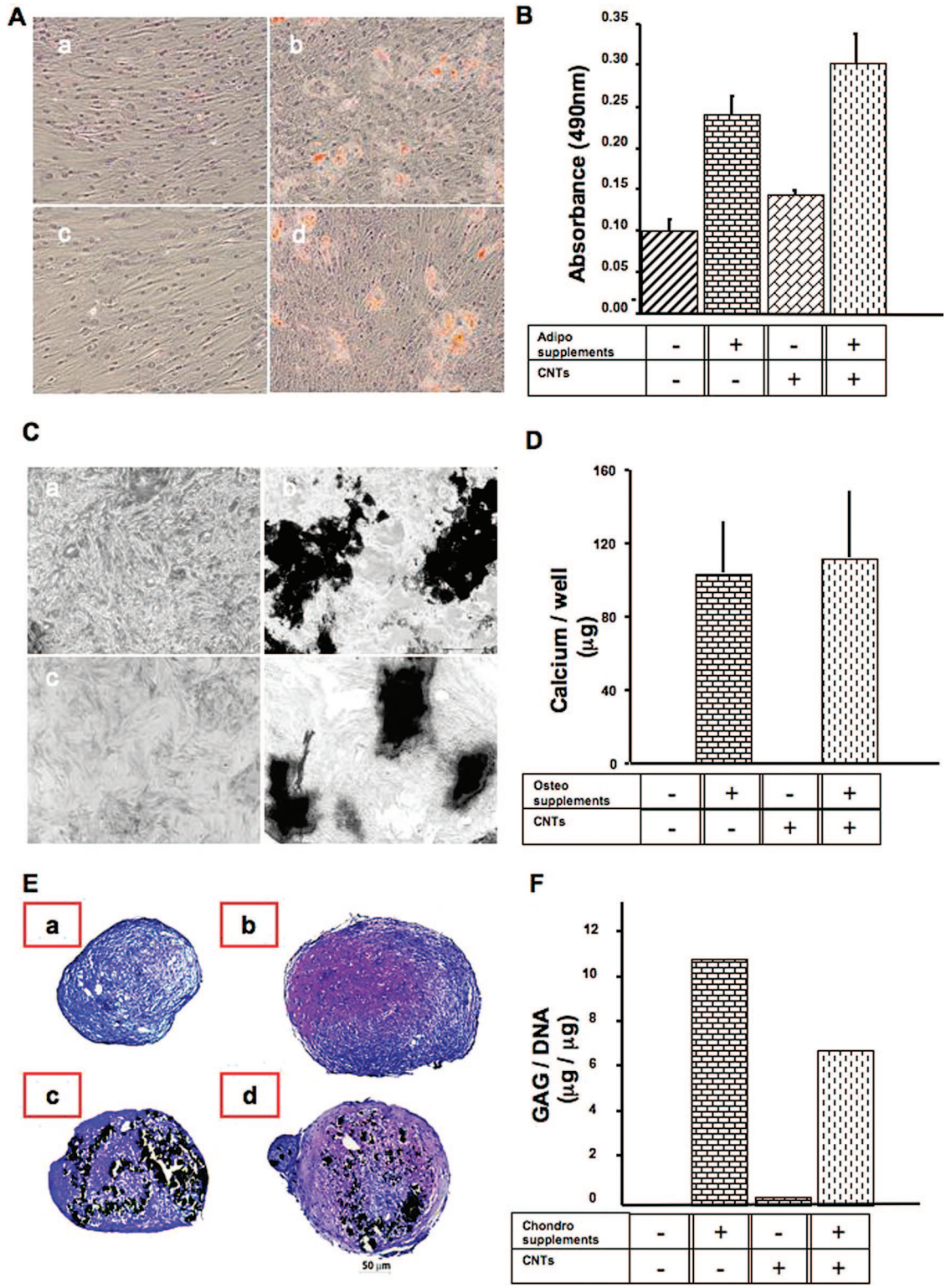

Figure 5. Effect of 0.032 mg/ml COOH-functionalized SWNT on hMSC phenotype. Adipogenesis (A) oil red O staining of the lipid vacuoles (mag. 10×), (B) extraction of oil red O. Osteogenesis (C) Von Kossa staining of calcium deposits (mag. 10×), (D) calcium assay. Chondrogenesis (E) toluidine blue staining of proteoglycans (GAG), (F) GAG assay. Error bars represent standard deviation of the mean ($n = 4$ donors).

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