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Evaluation of Cytocompatibility as Assessed by Genomic Stability of Canine induced Pluripotent Stem Cells Propagated on Carbon Nanotube Substrates

Tanmay Mondal, Pranay Konda, Kinshuk Das[1], Kuldeep Kumar, Sadhan Bag

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ABSTRACT

Background: Induced pluripotent stem cells (iPSC) are a type of pluripotent stem cell derived from adult somatic cells that have been genetically reprogrammed to an embryonic stem (ES) cell-like state through the forced expression of genes and factors important for maintaining the defined properties of ES cells. So far there are very few experiments that have been able to prove that nanomaterial-based scaffold can cultivate and maintain the iPSC as an alternative to feeder-free maintenance of iPSC.

Methods: The present experiment has given us fundamental information on *ex vivo* canine iPSC behavior on -OH functionalized single and multi-walled carbon nanotube (CNT) scaffold. Here in we evaluated the cytocompatibility of iPSC cultured on MEF feeder, OH-SWCNT and OH-MWCNT.

Result: We have seen very wonderful growth of ciPSC on CNTs similar to feeder. The cells were positive for alkaline phosphatase staining and expressed pluripotent markers. Cytotoxicity analysis revealed that -OH functionalized CNTs provide a milieu of low cytotoxicity. With this test we can interpret that -OH functionalized CNT can be used as xeno-free substrate to support the maintenance of iPSC in an undifferentiated state.

Key words: Canine induced pluripotent stem cells, DNA ladder assay, Functionalized carbon nanotube.

INTRODUCTION

Recent advances in the field of Induced Pluripotent Stem Cells (iPSCs) have opened up many gateways for therapeutic research. iPSCs are cells that are reprogrammed from somatic cells using a variety of transcription factors. They possess unique features of self-renewal and differ with different cell lineages. It can therefore replace embryonic stem cell (ESC) use and address various ethical issues regarding the use of embryos in research and clinics. While having promising important roles in various research, iPSCs can also be of great use in studying the molecular mechanisms of many diseases. In addition, iPSCs can be used to make patient-specific cells and apply to tissue injury sites for tissue regeneration. While adult stem cells of various sources are of preferred cell types but they have also several limitations. Easy isolation, patient specific origin and even derivation of Mesenchymal stem cells (MSCs) from these cells have made iPSC as the promising cell for therapeutic applications beside usefulness in disease modelling, drug testing, bioreactors and various agricultural applications such as transgenic animal production, animal cloning *etc.*

Pure cell population of iPSCs is very important for stem cell based therapy. Historically pluripotent stem cells including iPSCs were maintained on feeder cell layer (Takahashi *et al.,* 2007; Pan *et al.,* 2010). Now many new techniques have been discovered in feed free iPSC culture using extracellular matrix (vitronectin, fibronectin, *etc.*) which were standardized in different species (Amit and Itskovitz Eldor, 2006; Wu *et al.*, 2010; Nakagawa *et al.*, 2014).

Division of Physiology and Climatology, ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, Uttar Pradesh, India.
[1]Department of Veterinary Physiology, West Bengal University of Animal and Fishery Sciences, Kolkata-700 037, West Bengal, India.

Corresponding Author: Sadhan Bag, Division of Physiology and Climatology, ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, Uttar Pradesh, India. Email: bag658@gmail.com

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Although feeder cell and extracellular matrix is widely used, they are having many limitations (Villa-Diaz *et al.*, 2013). Therefore, synthetic materials that are easily available at low prices can be of excellent choice for propagation of pluripotent cells.

Carbon nanotubes (CNTs) of single walled (SWCNT) and multi-walled (MWCNT) are considered as one of the widely accepted biomedical materials owing to their unique mechanical, electrical, optical and magnetic properties as well as excellent compatibility with cells and tissues (Saito *et al.,* 2014). The effectiveness of CNT with OH, -COOH or -NH2 groups makes them highly bio-compatible. CNTs can be combined to strengthen the compound scaffold which enables better cell adhesion and tissue regeneration. Although CNT was first used a decade ago to grow nerve cells, they have been shown to support the proliferation and

differentiation of various adult stem cells (Li *et al*., 2012 and Holmes *et al*., 2016). In our previous research we have also shown that CNTs acting as a culture substrate serving as a positive signal for successful maintenance of canine MSC and iPSC (Das *et al*., 2017; Mondal *et al.,* 2021).

So far only two reports are available on CNT based culture of iPSCs. Akasaka *et al*., (2011) grew the mouse iPSC on SWCNT and MWCNT without any functionalization group and found that iPSC tended to grow less on the CNT coated dishes. Further, they have only checked the expression of early un-differentiation markers Alkaline phosphatase (ALP), Oct3/4 and Nanog but not the pluripotency of the cells grown on CNTs. Marina and Saavedra, (2014) also used the pristine CNT based substrate (hydrophilised and extracellular matrix coated CNT arrays) for self-renewal of human iPSC. They observed that hiPSC were unable to adhere to CNT arrays, underwent apoptosis and an insignificant small colonies were present.

We assumed that the functionalized carbon nanotube coated culture substrate could offer an appropriate micro environment for the proliferation and maintenance of ciPSCs without any profound genotoxicity and could be an alternative to feeder system of iPSC culture. In this study, canine iPSCs were cultured and maintained on -OH functionalized SWCNT and MWCNT coated surface while Mouse Embryonic Fibroblasts (MEFs) feeder layer was considered as control. We investigated the expression of pluripotency markers, colony morphology and alkaline phosphatase activity along with an assessment of the genotoxicity of CNTs. The study could lead to an understanding of the potential use of CNTs for iPSC's xeno-free, feeder-free culture system and could be tested for potential therapeutic applications in veterinary medicine.

MATERIALS AND METHODS

Preparation of functionalized carbon nanotube substrates

Hydroxyl (-OH) functionalized single-walled CNTs (SWCNTs, diameter 1-2 nm, length 0.5-2 µm) (Sisco Research Laboratory, India) and MWCNTs (Sigma, USA, diameter 9.5 nm, length 1.2 µm) powders were dispersed in ethanol (0.1 wt%) and finely separated by ultra-sonication. Homogenized carbon nanotube suspensions were then sprayed on the pre-heated coverslip with an airbrush. These coverslips were thoroughly air-dried and sterilized by ultraviolet (UV) radiations prior to cell culture. Field emission electron microscopy (FESEM) (Zeiss; Sigma; Germany) was performed to determine the surface morphology of carbon nanotube coated cover slips. Three dimensional images (3D) with different magnification were captured at an accelerating voltage of 10kV with varying distance (WDs). Surface roughness (Sq) was measured by optical 3D profilometry (contour GT-K 3D optical Microscope, USA).

Canine induced pluripotent stem cell

In all the experiments, frozen stock of ciPSC maintained at reproductive physiology laboratory, Physiology and Climatology Division, IVRI, Izatnagar were used.

Immunocytochemical staining

For immunolocalization of pluripotency markers Oct 4, Nanog, SSEA 4 and Tra 1-60 and Tra 1-81 (Santacruiz, USA, Table 1A), ciPSCs of 8th passage were cultured onto feeder as well as CNT substrates. The cells were fixed in paraformaldehyde (PFA) at 4°C for 20 min. and permeabilized with 0.3% Triton X-100 for 20 minutes. The non-specific sites were blocked using 5% BSA for 40 min and then incubated overnight at 4°C with primary antibodies followed by FITC-conjugated secondary antibodies for 4 hr. at room temperature. The cells were counterstained with the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) and stained colonies were observed under fluorescent microscope (Carl-Zeiss Apotome, Germany).

Alkaline phosphatase staining

ALP staining was performed using commercial kit (Sigma-Aldrich) according to the manufacturer's protocol. The ciPSCs were fixed with citrate-acetone-formaldehyde solution and then stained with alkaline phosphatase staining reagent (Naphthol/fast red violet). Colonies positive for ALP activity were stained pink-red color which was recorded under phase contrast microscope (Olympus, Japan).

Expression of pluripotency associated genes

We performed RT-PCR analysis of the ciPSCs to determine the expression of pluripotency associated genes such as Oct 4, Nanog, Klf 4, Sox 2, FoxD3 and cMyc, Total RNA was extracted from the cells using an RNeasy Kit (Qiagen, Valencia, CA, USA) followed by reverse-transcription using a cDNA synthesis kit (iScript, BioRad). RT-PCR analysis (Bio-Rad, CFXManager™ Software) was performed using the DyNAmo Color Flash SYBR green qPCR kit (thermo fisher scientific, USA) using canine-specific primers (Table 1B). The PCR products were size fractionated by 2% agarose gel electrophoresis.

In vitro differentiation of ciPSCs into three germ layers

The differentiation ability of ciPSC was examined by hanging drop culture. Briefly, single cell suspension of ciPSCs (2.5×10^4 cells/ml) were prepared in differentiation medium containing IMDM (high glucose), 100 mM α-mercaptotoethanol, 10% FBS, 1% NEAA. From this cell suspension, 20 µl was pipetted on to the lid of a tissue culture dish and then inverted over its bottom dish containing 5 ml of phosphate-buffered saline. On day 3, the embryo bodies (EBs) was transferred on a gelatin-coated coverslip as well as onto CNT substrates and grown up to day 12, with periodical changing of the differentiation medium. There after, total RNA was extracted from the differentiated cells to check the expression of three representative genes of each germ layer *viz.* TUBB 3 for ectoderm, GATA 2 for mesoderm and CXCR 4 for endoderm. PCR products were size fractionalized and bands were illuminated under gel documentation system (Bio-Rad).

Table 1A: Primary and secondary antibody list.

Antigen	Antibody host and type	Dilution	Source
Nanog (W-18)	Goat polyclonal IgG	1:100	Santacruiz Biotechnology
OCT3/4 (C-10)	Mouse monoclonal IgG_{2b}	1:100	Santacruiz Biotechnology
SSEA-4 (MC813)	Mouse monoclonal IgG_1	1:100	Santacruiz Biotechnology
TRA1-60 (TRA-1-60)	Mouse monoclonal IgM	1:100	Santacruiz Biotechnology

Table 1B: Primer list.

Target gene	Primer sequence 5′ to 3′	Annealing temperature (°C)	Amplicon size (bp)	Accession no.
GAPDH	F- CCATCTTCCAGGAGCGAGAT	55	97	Vieira *et al.*, 2010
	R- TTCTCCATGGTGGTGAAGAC			
c-Myc	F- CACCCATCAGCACAACTACG	56	172	X95367.1
	R- GTTGTGTGTTCGCCTCTTGT			
Nanog	F- GAGAGAACAGCGAGGAAGGA	55	135	XM_022411387.1
	R- TTGCATCTGCTGGAGACTGA			
FOXD3	F- CCGCCACAACCTCTCGCTCAA	59	93	XM_003434606.3
	R- TGCGGGTCCAGGGTCCAGTAGT			
SOX2	F- CCCCGTGGGTTACCTCCTCCTCCC	61.3	148	XM_005639752.3
	R- GCCGCTCTGGTAGTGCTGGGACA			
KLF-4	F- CCTCGCCTTACACATGAAGAG	61.8	124	XM_038682371.1
	R- GGAGGGAAGACAGTGTGAAAG			
GATA-4	F- GGCCCTGAAGCTCTCCCCACAAG	58	93	XM_014107460.2
	R- AAGGCCAGGCTGTTCCAAGGGTC			
TUBB-3	F- AGCCAAGTTCTGGGAAGTCA	57	238	Wilcox et al.(2011
	R- CCCACTCTGACCAAAGATGAA			
OCT4	F- TGCCCGAAGCCCACCCTGC	60	94	XM_538830.3
	R- GCCGACGATTGCAGAACCACACT			

DNA ladder analysis by gel electrophoresis

To determine the genomic DNA damaging effect, ciPSC colonies were seeded onto MEF feeder as control as well as OH-SWCNT and OH-MWCNT scaffolds under standard culture conditions to 6 well plates and cultured for 3 days. The DNA damage of ciPSCs in the control and scaffolds was determined by DNA ladder analysis by gel electrophoresis on day 3 of culture. DNA was extracted from ciPSC colonies by accutase mediated enzymatic disaggregation and transferred 5×10^5 cells to 1.5 ml sterile microcentrifuge tubes. Cell suspension was centrifuged at 1500 rpm in an Eppendorf table top centrifuge for 5 minutes at 4°C and supernatant was discarded. Then 20 µl of TES [N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid-N-tris methyl-2-ammonioethanesulfonate] lysis buffer was added and proper mixing of cell pellet with TES lysis buffer was done by stirring with a wide-bore pipette tip. 10 µl of RNase cocktail was added and mixed well by flipping the tip of the tube. Simultaneously, 10 µl of proteinase K was added to solution and mixed well by flipping the tip of the tube and incubated at 50°C for at least 90 minutes. Further, 5 µl of 6X DNA loading buffer was added and DNA samples loaded into dry wells of a 1.5% agarose gel in 1X TAE containing 0.5 µg/ml ethidium bromide. Gel electrophoresis was performed at a low voltage of 35 V for ~4 hours. DNA ladders were finally visualized by a UV light source and documented by photography. Apoptotic cells formed a distinct DNA ladder.

RESULTS AND DISCUSSION

The physical appearances of two varieties of CNT scaffolds were observed by FESEM. CNTs formed thin network over the cover slip. In higher magnification, an individual CNT bundle was visible and formed a mesh-like network throughout the cover slip (Fig 1A). Optical profilometry imaging showed that a three dimensional (3D) surface topography had been created on the cover slips where the root mean-square roughness (Sq) was calculated as 1.6 and 1.4 times higher in SWCNT and MWCNT, respectively (Fig 1B). SWCNT and MWCNT bundle were well defined and formed a continuous network throughout the coverslips as evident by FESEM that is representative of the nano roughness or nano features made available for the cells to interact at the cell-material interface. In the case of colony morphology, we have noticed a round shaped, uniformly concentric, compact and raised colony of ciPSC in different passages. These are the characteristics of a typical undifferentiated induced pluripotent stem cell that have been maintained on CNT substrates. This indicates that while the CNT surface appears to be a favorable cue, it does not impede the growth and colonization of ciPSCs. It has been established that cell adhesion, proliferation and its cytoskeleton adaptation are largely influenced by the roughness and surface chemistry of the nanotopograph of the culture substrate. It has been established that cell

adhesion, proliferation and its cytoskeleton orientation are largely influenced by the roughness and surface chemistry of the nanotopograph of culture substrates (Das *et al.*, 2017; Mondal *et al.*, 2021).

Each colony was splitted into 4 to 8 pieces with the help of tuberculin needle under stereo zoom microscope and subsequently propagated on gamma irradiated MEF feeder layer as well as on both OH-SWCNT and OH-MWCNT scaffolds under standard culture conditions. After seeding, the colonies were attached and spread eventually in control and CNT scaffolds with compact roundness like morphology of pluripotent stem cells (Fig 1C). The compact round morphology of typical iPSC colonies were maintained on all the surfaces even after 72 hours of incubation (Fig 2D) The pluripotency of iPSC is defined in terms that whether the cells have the capability to differentiate into three germ layers *in vitro* or *in vivo* (Mahapatra *et al.,* 2016). ciPSC are unique in their ability to express pluripotency markers and higher ALP activity (Baird *et al.,* 2015). In our study, we found positive expression of pluripotency markers *viz.* Nanog, Oct 4, SSEA and Tra-1-60 (Fig 2C), as well as strong AP expression while culturing of ciPSCs on feeder, OH-SWCNT and

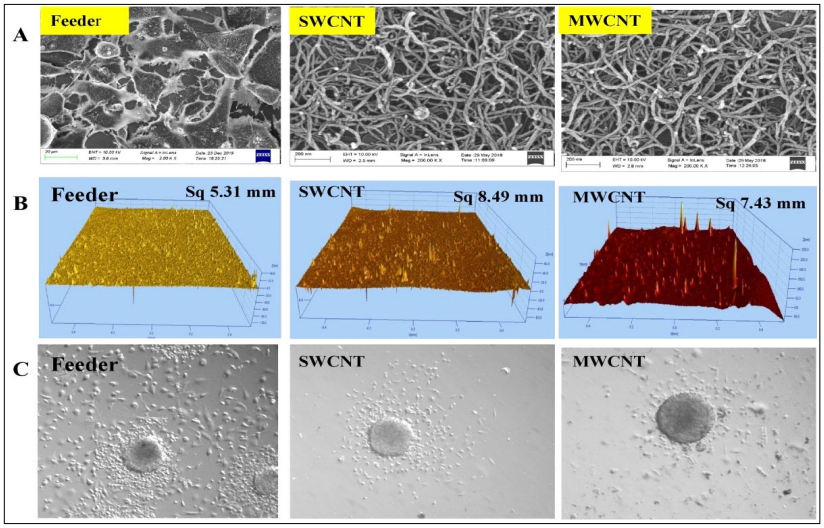

Fig 1: Maintenance of ciPSC colonies on feeder and two types of CNT substrates.

Notes: (A) FESEM images of the feeder and CNT substrates (Feeder scale 100 µm). (B) Optical profilometry images of CNT substrates (C) Phase contrast images of colony morphology at different passages maintained on the feeder and on CNT substrates, Scale: 50 µm.

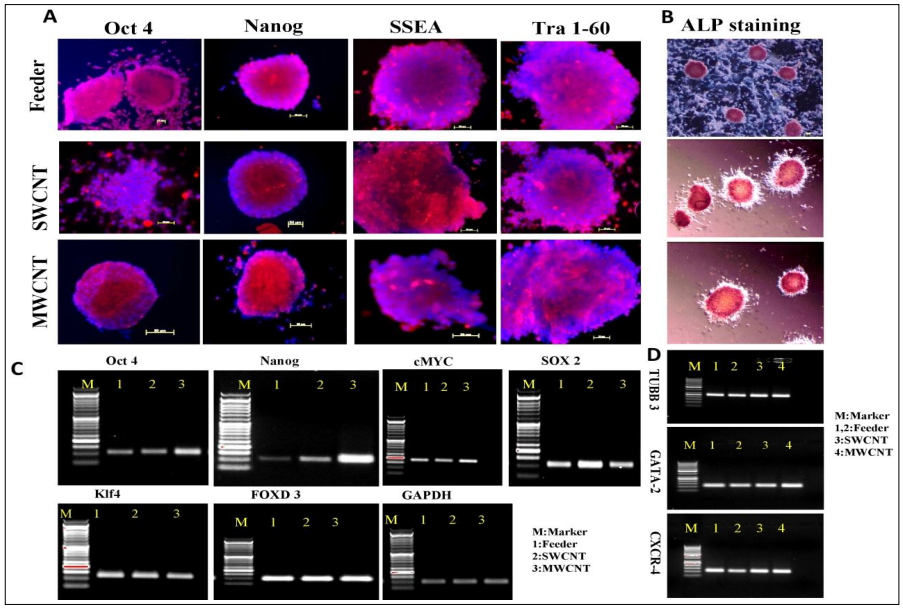

Fig 2: Maintenance of the pluripotency of ciPSCs on CNT substrates.

Note: (A) Immunolocalization of pluripotency markers Oct 4, Nanog, SSEA4 and Tra-1-60, Scale: 50 µm. (B) Alkaline phosphatase staining, Scale: 100 µm. (C) Expression profile of pluripotency genes on different culture surfaces. (D) Expression of gene markers after *in vitro* three lineage differentiation of ciPSCs on different culture surfaces.

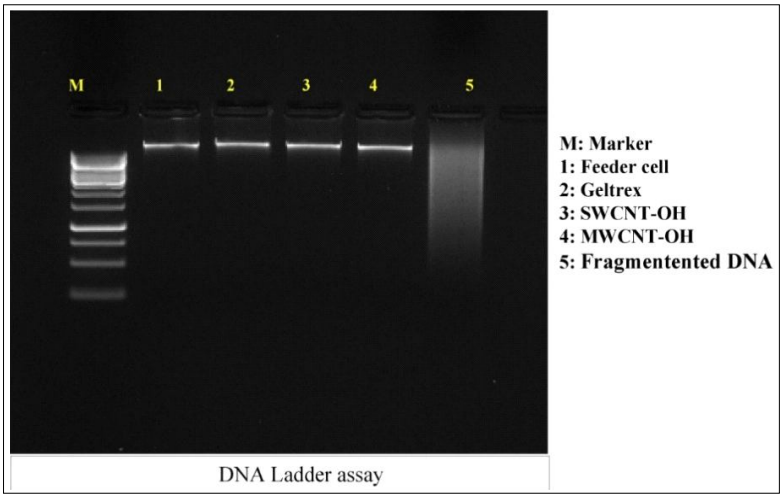

Fig 3: DNA ladder assay for detection of DNA damages of ciPSCs cultured on different substrates. Feeder (Lane 1), Geltrex (Lane 2) SWCNT (Lane 3), MWCNT (Lane 4), (Lane 5) +ve control and bands were detected on 0.8% agarose gel electrophoresis. Lane M-Ladder (1Kb).

OH-MWCNT (Fig 2B). This is in agreement with Akasaka *et al.* (2011) and Brunner *et al.* (2014) that culturing of mouse iPSCs and human ESC on non-functionalized CNTs continued to express pluripotency markers.

The attached ciPSCs were immunopositive for pluripotency specific markers Oct 4, Nanog, SSEA and Tra-1-60 (Fig 2A).

To investigate whether CNT substrates induced cell death by apoptosis, DNA laddering assay was done on agarose gel. Breakages of DNA strand in apoptotic cells can be detected by this assay. Ladder pattern of inter nucleosomal fragmentation was not observed in the DNA of ciPSCs maintained onto feeder and CNTs whereas, a fragmented ladder pattern was noticed when ciPSC were treated with H_2O_2 for 30 min at 60°C, taken as positive control (Fig 3). These results pointed out that functionalized CNT substrates did not cause significant DNA damage in ciPSCs. The DNA ladder assay is a simple, sensitive and rapid method for estimating apoptotic changes in cell populations. This has several recompenses over other tests, like exclusion of laborious and multi-step manipulations during DNA extraction, no pre-labeling of the cells is required, *etc.* Also, this assay allows working with cell lysates and dose not requires any special laboratory equipment. The results indicated that CNT substrate could be an effective cytocompatible material for culture of canine iPSCs.

CONCLUSION

Now-a-days, the toxicity profiling of nano-material is a highly demanded research area. We observed that ciPSCs can be maintained onto the OH-functionalized SWCNT and MWCNT substrates alleviating the need of feeder based maintenance. The ciPSCs were able to maintain their colony morphology and also expressed pluripotency markers including high ALP activity. Our genotoxicity study indicated that these substrates did not have profound genotoxic effect on ciPSCs. Thus, it can be concluded that OH-CNT coating may acts as a substrate for feeder free culture of canine iPSCs.

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Conflict of interest: None.

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