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Dose-dependent *In Vitro* Cytotoxic Effects of Phosphorus Doped Graphene Oxide Titanium Oxide Nanocomposite (P-GO-TiO_2 NC) on Caprine Wharton's Jelly Derived Mesenchymal Stem Cells (WJ-MSCs) and Erythrocytes

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ABSTRACT

The present investigation was conducted to evaluate the dose dependent (100, 50, 25, 10 and 0 µg/ml) cytotoxic effects of phosphorus doped graphene oxide titanium oxide nanocomposite (P-GO-TiO_2 NC) on caprine Wharton`s jelly-derived mesenchymal stem cells (WJ-MSCs) by observing cell morphology, viability, growth kinetics, PDT (population doubling time), MTT (Tetrazolium dye 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) and hemolysis assay using caprine erythrocytes. The caprine WJ-MSCs exhibited fibroblastoid morphology which was changed significantly as cells detached from culture surface at 100 and 50 µg/ml doses while 25 and 10 µg/ml did not altered cell morphology after 48 and 72 hrs exposure as compared to control. Viable cell number after 48 and 72 hrs of incubation with P-GO-TiO_2 NC in 25 µg/ml dose was significantly ($P<0.01$) higher while it was significantly ($P<0.01$) lower at 100 and 50 µg/ml doses as compared to 10 µg/ml and control. Caprine WJ-MSCs PDT was significantly ($P<0.05$) increased at 100 and 50 µg/ml doses with significantly ($P<0.05$) altered shape of growth curve as compared to 25, 10 and 0 µg/ml doses of P-GO-TiO_2 NC. Metabolically active caprine WJ-MSCs were significantly ($P<0.01$) increased at 25 and 10 µg/ml doses, while it was significantly ($P<0.01$) decreased at 100 and 50 µg/ml doses as compared to control, which was determined by MTT assay. Erythrocyte hemolysis assay revealed that P-GO-TiO_2 NC significantly ($P<0.05$) destructed the caprine erythrocytes at 100 µg/ml doses as compared to 50, 25, 10 and 0 µg/ml doses. It is concluded that P-GO-TiO_2 NC at 25 and 10 µg/ml doses are biocompatible and enhance caprine WJ-MSCs growth as compared to 100 and 50 µg/ml doses.

Keywords: Graphene oxide, Titanium oxide, Nanocomposite, Stem cells, Cytotoxicity

In biomedical sciences, the nanotechnology is used for drug discovery and delivery, imaging, separation and purification of biological molecules and cells, proteomics and genomics, disease diagnosis and regenerative medicine. Stem cells are multipotent cells and differentiate into numerous tissue (Kolf *et al*., 2007) and can be isolated from different sources such as developing embryo, bone marrow, peripheral blood, adipose tissue, liver and fetal adnexa (cord blood, amniotic fluid, amniotic sac and Wharton's jelly) in different species. Wharton's jelly of the fetal umbilical cord is a good source of mesenchymal stem cells (MSCs) (Batsali *et al*., 2013) and Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) can be used to study *in vitro* cytotoxicity of nanomaterials. Recently, stem cell nanotechnology has evolved as a novel field to utilise stem cells (Seyedjafari *et al*., 2010) for generation of new tissues / organ to treat numerous regenerative diseases in human as well as farm animals. The nanosize of materials, which is the three dimensional matrices that regulate the stem cell growth and differentiation due to their intrinsic properties such as nanotophography, roughness, energy, extracellular forces, magnetic and optical effects (Krishna *et al*., 2016). Several nanomaterials were studied in stem cell research to apply mainly in regenerative medicine as the three dimensional scaffolds (Yadegar *et al*., 2015), stem cell differentiation (Guo *et al*., 2017), intracellular

delivery of DNA and RNA (Park *et al*., 2011), *in vivo* stem cell delivery (Nguyen, 2013) and tracking (Moghimi *et al*., 2005) and stem cell therapy in cancer (Wang *et al*., 2014). However, nowadays, the identification of good and reliable source of stem cells and *in vitro* maintenance of stem cells with nanomaterials and their *in vivo* delivery and transplantation is great challenges in stem cell research. *In vitro* cytotoxicity of nanomaterials is pre-requisite and certain reports indicated that the dose and exposure time are vital factors in biocompatibility of nanomaterials in stem cells (Li *et al*., 2014) and blood cells (Ghosh *et al*., 2013). Recently, the bioactive ceramics, organic and inorganic nanomaterials are considered as new tools in drug delivery in antibacterial and anticancer therapy, imaging and tissue engineering applications (Dillip *et al*., 2017). Organic nanomaterial, graphene oxide (GO), is oxygenated derivative of graphene which is atom thick carbon monolayer arranged in two dimensional honeycomb structure (Allen *et al*., 2010).

However, certain reports show *in vitro* cytotoxicity of graphene based nanomaterials (GBNs) in caprine WJ-MSCs at moderate (50 µg/ml) and high doses (100 µg/ml) (Dar *et al*., 2015; Gade *et al*., 2015), SiO_2 nanoparticle in RAW 264.7 cells (Park *et al*., 2011), SiO_2, N-SiO_2 and Ag-SiO_2 nanoparticles in hBM-MSCs and hAD-MSCs (Tarpani *et al*., 2016), TiO_2 in human bronchiolar epithelial cells, 3T3 fibroblast and RAW 264.7 cells in high doses (100 and 1000 µg/ml) (Sohaebuddin *et al*., 2010). However, some researchers reported the biocompatibility and stimulating effect of GBNs in caprine BM-MSCs (Elkhenany *et al*., 2015), MC3T3-E1 cells (Lee *et al*., 2015; Nishida *et al*., 2016), hAD-MSCs (Nair *et al*., 2015), murine haemangioblasts (Alegria *et al*., 2016). In addition, heteroatoms doped graphene derivatives exploit their unique properties (Wang *et al*., 2012) and these doped graphene nanocomposites are not studied in stem cell research. Hence, in the present study, we hypothesized that the phosphorus doped GO and their nanocomposite with TiO_2 nanomaterial (P-GO-TiO_2 NC) may reduce cytotoxicity of GO exhibit dose dependent biocompatibility in caprine WJ-MSCs and erythrocytes.

MATERIALS AND METHODS

Chemicals, reagents and plastic wares used in this study were procured from Hi-Media Lab. Pvt. Ltd., Mumbai (India) and P-GO-TiO_2 NCs were supplied by Department of Physics, Banaras Hindu University, Banaras, Uttar Pradesh (India).

Isolation and culture of caprine WJ-MSCs

The present study was conducted in Stem cell culture laboratory at Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Anjora, Durg (Chhattisgarh), India with the approval of Institutional Animal Ethics Committee.

Caprine gravid uteri (~45 days, n=4) were collected aseptically from the local abattoir and were transported to the Stem cell culture laboratory within 1 hr of collection. The collected uteri were washed with normal saline and processed immediately to isolate WJ-MSCs as per the procedure described by Babaei *et al*. (2008) with some modifications. In brief, gravid caprine uterus opened and 2.0 to 2.5 cm long fetal umbilical cord (UC) cut and washed in phosphate buffer saline (PBS) and UC incised longitudinally and blood vessels removed carefully. Wharton's jelly mesenchymal connective tissues were isolated, washed with phosphate buffer saline ((PBS) and in Dulbecco's modified eagle's medium (DMEM) supplemented with fetal bovine serum (FBS) (15%) and antibiotic and antimycotic solution (1%), sliced in small explant (1-2 mm^2). Wharton's jelly explants (4-5) were seeded in a tissue culture flask and maintained at 37ºC with 5% CO_2. Caprine WJ-MSCs were observed on day 3 on periphery of jelly explant and jelly explants were removed on day 5 and cells were maintained for 12-14 days before passage. Cell culture media was changed initially on day 5 of seeding and thereafter every 4^{th} day until culture retained. Third passage WJ-MSCs were used for cytotoxicity assays.

Isolation of caprine erythrocytes

In Ethylenediaminetetraacetic acid (EDTA) coated tube, 5.0 ml blood was collected through jugular vein puncture from adult caprine and erythrocytes were isolated from the whole blood as per the method described by Vinjamuri *et al*. (2015) and used immediately for hemolysis assay.

Cytotoxicity assays

Total 5 treatment groups of 100, 50, 25, 10 and 0

(control) µg/ml doses of P-GO-TiO_2 NC in DMEM were constituted to study *in vitro* cytotoxicity in caprine WJ-MSCs and erythrocytes. Cell morphology, viability, PDT, growth kinetic and MTT of WJ-MSCs and hemolysis of erythrocytes were carried out in triplicate.

Cell morphology assay

Caprine WJ-MSCs were treated with different doses of P-GO-TiO_2 NC and any morphological alterations in cells were observed after 48 and 72 hrs using the inverted microscope (Nikon Diaphot 300) and morphological changes in caprine WJ-MSCs were compared with control group.

Cell viability

Caprine WJ-MSCs were seeded at the density of 5×10^4 cells / well in 24 well cell culture plates and exposed to different doses of P-GO-TiO_2 NC and cell viability was assessed after 48 and 72 hrs post exposure by trypan blue dye exclusion technique as described by Bregoli *et al*. (2009).

Cell growth kinetic

Caprine WJ-MSCs growth characteristics upon exposure to different doses of P-GO-TiO_2 NC were studied for 14 days. Caprine WJ-MSCs were plated at density $1x10^4$ cells / well in 24 cell culture plates and cell culture media containing respective concentrations of P-GO-TiO_2 NC was changed at every 4^{th} day. Cells from each 24 well cell culture plate were harvested every after 48 hrs intervals and counted using hemocytometer (Neubauer's cell counting chamber) and growth curves were plotted and compared with control.

Population doubling time (PDT)

Caprine WJ-MSCs were incubated with P-GO-TiO_2 NC at different doses in 24 well cell culture plates at cell density $1x10^4$ / well for 3 days and at every 24 hrs interval cells from each plate were harvested and counted using a hemocytometer (Neubauer's cell counting chamber) and PDT was calculated using the equation described by Gade *et al*. (2015),

$$PDT = \text{Culture time (CT)}/\text{Cell doubling (CD)}$$

where, $CD = \log (NH/NI)/ \log 2$,

N_H is harvested cell number and N_I is initial cell number

MTT (Tetrazolium dye 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay

Colorimetric assay was performed to evaluate cytotoxicity of P-GO-TiO_2 NC in caprine WJ-MSCs using MTT Cell Assay Kit (Hi-Media Lab. Pvt. Ltd., Mumbai)as per the instructions of manufacturer. Briefly, 100 µl freshly harvested caprine WJ-MSCs cultured with different doses of P-GO-TiO_2 NC in wells of 96 well cell culture plate for 24 hrs and cell monolayer treated with MTT reagent and incubated for 4 hrs in CO_2 incubator. Media aspirated with care and 100 µl of solubilization solution was added in each test well and gentle stirring was done to dissolve MTT formazan crystals completely and absorbance was read thrice at 620 nm on ELISA plate reader (MULTISKAN EK, Thermo Scientific).

Hemolysis assay

Caprine erythrocytes were incubated with different doses of P-GO-TiO_2 NC in Dulbecco's phosphate buffer saline at 37°C in storage incubator for 4 hrs. All samples were centrifuged and 100 µl supernatant transferred with care in flat bottom 96 wells tissue culture plate and absorbance was read thrice at 492 nm in ELISA plate reader (MULTISKAN EK, Thermo Scientific) and hemolysis (%) was calculated using following formula,

$$\text{Hemolysis \%} = \frac{\text{Absorbance value of test sample} - \text{Absorbance value of negative control}}{\text{Absorbance value of positive control}} \times 100$$

Statistical Analysis

All data recorded in the present study are expressed as mean ± standard error (S.E.) values and One-way analysis of variance (ANOVA) was applied using IBM SPSS Statistics 25 and values of $P<0.01$ and $P < 0.05$ are considered to be statistically significant.

RESULTS AND DISCUSSION

Caprine WJ-MSCs were isolated from Wharton's jelly of fetal UC and cultured in DMEM supplemented with FBS (15%) and antibiotic and antimycotic solution (1%). Caprine WJ-MSCs protruded from Wharton's jelly explants and grew well on day 3, spindle shaped cells with large nucleus and exhibited distinct fibroblastoid morphology on day 7 with generated colonies and reached confluent on day 14 (Fig. 1). Similar findings were reported earlier in caprine WJ-MSCs (Moshrefi *et al*., 2010; Dar *et al*., 2015; Gade *et al*., 2015) and MSCs isolated in caprine from fetal adnexa (Somal *et al*., 2016) and bone marrow (Elkhenany *et al*., 2015; Towseef *et al*., 2017).

Caprine WJ-MSCs morphology did not change by P-GO-TiO_2 NC at 25 and 10 µg/ml doses but, 100 and 50 µg/ml doses altered cell morphology distinctly as compared to control after 48 and 72 hrs exposure (Fig. 2). In addition, caprine WJ-MSCs were detached from surfaces and floated in media after 48 and 72 hrs exposure to high doses (i.e. 100 and 50 µg/ml) while, lower doses (i.e. 25 and 10 µg/ml) of P-GO-TiO_2 NC stimulates growth and proliferation which showed normal fibroblastoid morphology like control (Fig. 2).

In the present study, P-GO-TiO_2 NC significantly altered caprine WJ-MSCs morphological characteristics at high doses but, lower doses are biocompatible and stimulated

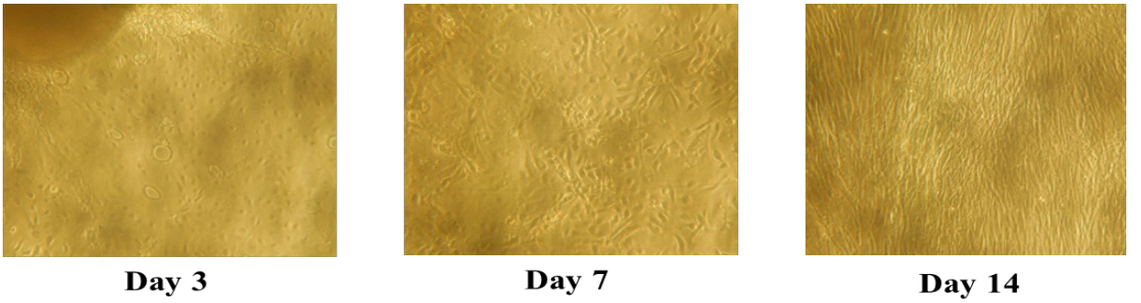

Fig. 1: Caprine WJ-MSCs at different interval

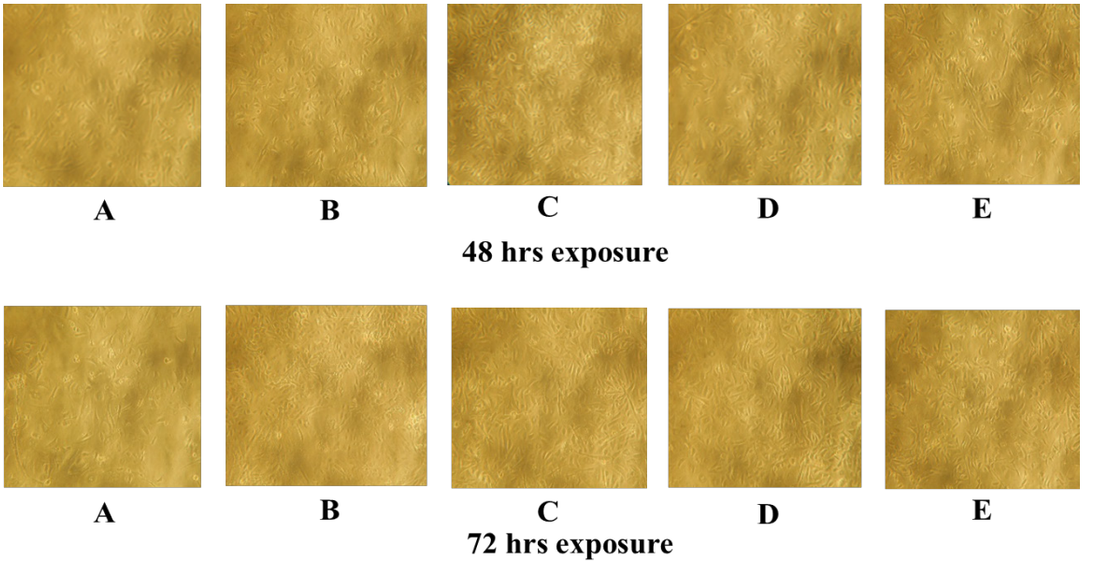

Fig. 2: Morphology of caprine WJ-MSCs, which were exposed to different doses of P-GO-TiO_2 NC for 48 and 72 hrs (A-100 µg/ml, B -50 µg/ml, C-25 µg/ml, D-10 µg/ml and E-0 µg/ml)

growth. Earlier similar findings were reported in caprine WJ-MSCs exposed to graphene quantum dots (GQDs) (Dar *et al*., 2015) and GO-Fe_2O_3 NC (Gade *et al*., 2015). Human neural stem cells (Park *et al*., 2011), human MSCs (Nayak *et al*., 2011) and caprine BM-MSCs (Elkhenany *et al*., 2015) that were cultured on graphene coated glass substrates exhibited similar morphology in lower doses of P-GO-TiO_2 NC (25 and 10 µg/ml). Exposure time (24 and 48 hrs) and dose (5-600 µg/ml) dependent morphological alterations were observed in rat pheochromocytoma cells (PC12) under exposure of SWCNTs (Wang *et al*., 2011). However, human BM-MSCs (Qu *et al*., 2016) cultured on Ti based SiO_2 substrates synthesized under O_2, N_2 and argon environment and displayed typical fibroblastoid morphology and did not differ significantly.

Caprine WJ-MSCs cell viability after 48 and 72 hrs treatment with P-GO-TiO_2 NC at 100 and 50 µg/ml doses significantly ($P<0.01$) decreased while, 25 µg/ml dose of P-GO-TiO_2 NC significantly ($P<0.01$) increased viable cell number as compared to 10 µg/ml dose and control (Table 1) with no significant difference between 48 and 72 hrs treatments. Similar results were reported earlier as significant ($P<0.01$) decrease in caprine WJ-MSCs viability on 48 hr exposure with high (100 µg/ml) and moderate doses (50 µg/ml) of GQDs (Dar *et al*., 2015) and GO-Fe_2O_3 NC (Gade *et al*., 2015) as compared to low dose (10 µg/ml) and control (0 µg/ml). MWCNTs in 80 µg/ml dose significantly decreased RAW-264.7 cell viability (Szczypta *et al*., 2012) and reduced human osteoblast cell density on sintered graphite (Czarnecki *et al*., 2008). However, Nayak *et al*. (2011) reported non significant difference in human MSCs cultured on graphene coated SiO_2, Polymethylsiloxane, Polyethelene trephthalate and uncoated glass substrate. Similar cytotoxic effect of TiO_2, SiO_2 and MWCNTs in high doses (100 and 1000 µg/ml) were demonstrated in 3T3 fibroblast, RAW 264.7 and human bronchiolar epithelial cells (Sohaebuddin *et al*., 2010).

Calibrated growth curves of caprine WJ-MSCs exposed to different doses of P-GO-TiO_2 NC were plotted and compared with control (Fig. 3). Caprine WJ-MSCs in control group followed normal growth pattern and exhibits initial slow growth of lag phase followed by exponential growth of log phase and stationary phase with declined growth rate. In the present study caprine WJ-MSCs (control) similarly grown with similar PDT as reported earlier in caprine WJ-MSCs (Pratheesh *et al*., 2014; Dar *et al*., 2015; Gade *et al*., 2015) and buffalo BM-MSCs (Gade *et al*., 2012). High doses (100 and 50 µg/ml) of P-GO-TiO_2 NC significantly ($P<0.05$) altered shape of growth curve (declined shape) with significant ($P<0.05$) increased PDT of caprine WJ-MSCs as compared to 25, 10, 0 µg/ml dose (Table 1 and Fig. 3) while, significant ($P<0.05$) altered (inclined shape) growth curve at 25 µg/ml dose as compared to all doses of P-GO-TiO_2 NC. However, non significant decrease in caprine WJ-MSCs PDT at 25 and 10 µg/ml doses were observed as compared to control (Table 1).

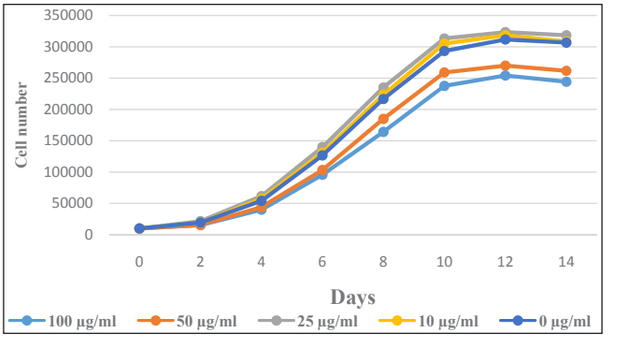

Fig. 3: Caprine WJ-MSCs growth curve under exposure of P-GO-TiO_2 NC at different doses

Present findings are in accordance with previous reports where GQDs (Dar *et al*., 2015) and GO-Fe_2O_3 NC (Gade *et al*., 2015) were treated in caprine WJ-MSCs, MWCNTs and SWCNTs in A549 lung epithelial cells in 100 µg/ml (Wadhwa *et al*., 2011), dose dependent cytotoxicity of graphene and CNTs in RAW-264.7 cells (Figarol *et al*., 2015). However, Ti substrates promotes human osteoblast adhesion and proliferation (Webster and Ejiofor, 2004), TiO_2 (<100 µg/ml) and Ti-GO were biocompatible in human bronchiolar epithelial cells (Sohaebuddin *et al*., 2010) and human BM-MSCs respectively (La *et al*., 2014) and expressed osteogenic markers on day 14. Cell doubling time was increased due to reduced cell growth and proliferation rate and increased PDT of caprine WJ-MSCs treated with P-GO-TiO_2 NC in high doses (100 and 50 µg/ml) is similar with the earlier findings reported in caprine WJ-MSCs exposed to GQDs (Dar *et al*., 2015) and GO-FeO_2O_3 NC (Gade *et al*., 2015).

In MTT assay mean absorbance values of caprine WJ-MSCs treated with P-GO-TiO_2 NC at 100 and 50 µg/ml

Table 1: Effect of P-GO-TiO_2 NC on caprine WJ-MSCs viability, population doubling time, absorbance values and erythrocytes hemolysis. (Mean ± S.E., n= 3)

Sl. No.	Doses (µg/ml)	Cell viability (%) after 48 hrs	Cell viability (%) after 72 hrs	PDT (hrs)	Absorbance values	Hemolysis (%)
1	100	74.81±0.37^{a}	73.20±0.35^{a}	49.74±2.62^{b*}	0.047±0.0001^{a}	2.80±0.52^{b*}
2	50	76.78±0.38^{b}	74.62±0.25^{a}	48.44±3.01^{b*}	0.051±0.0007^{b}	1.91±0.35^{ab*}
	25	89.47±0.52^{d}	88.38±0.26^{c}	38.45±0.69^{a*}	0.065±0.0010^{d}	1.74±0.12^{a*}
4	10	83.86±0.79^{c}	82.70±0.29^{b}	40.66±0.83^{a*}	0.063±0.0003^{d}	1.69±0.03^{a*}
5	0	82.44±0.31^{c}	80.58±0.71^{b}	41.50±0.83^{a*}	0.057±0.0012^{c}	1.34±0.13^{a*}

Mean values bearing superscript in column differed significantly from each other. (P<0.01) and (*P<0.05)

doses were significantly (P<0.01) decreased while, in 25 and 10 µg/ml doses absorbance values were significantly (P<0.01) increased as compared to control (Table 1). Increased absorbance values indicate more metabolically active cells due to lactate dehydrogenase activity and it confirms as increased proliferation rate caprine WJ-MSCs at 25 and 10 µg/ml doses of P-GO-TiO_2 NC. As previously reported significant (P<0.01) decrease in absorbance values in caprine WJ-MSCs in moderate (50 µg/ml) and high doses (50 µg/ml) of GQD (Dar *et al*., 2015) is similar with these findings. But, non cytotoxic effect of GO film reported earlier in caprine BM-MSCs (Elkhenany *et al*., 2015), human AD-MSCs (Nair *et al*., 2015) and GO significantly (P<0.01) increased endothelial (TIE2) and haematopoietic (CD41) cell markers in murine haemangioblasts (Alegria *et al*., 2016) and promoted proliferation of L929 and MG63 cells (Li *et al*., 2014). However, dose dependable (5-600 µg/ml) cytotoxicity of SWCNTs demonstrated in rat pheochromocytoma cells (Wang *et al*., 2011) but, TiO_2 nanobelts induced severe cytotoxicity at 10 µg/ml dose as compared to SWCNTs in 100 µg/ml dose in macrophage like THP-1 and small airway epithelial cells (Tilton *et al*., 2014) on 24 hr exposure. Recently, Guo *et al*. (2017) reported similar results where reduced graphene oxide (rGO) nanostructured microfibers and graphene film significantly increased rat neural stem cells proliferation rate and expressed neuronal and glial cell surface markers.

Caprine erythrocytes significantly (P<0.05) ruptured with treatment of P-GO-TiO_2 NC at high dose (100 µg/ml) as compared to 50, 25, 10 µg/ml dose and control after 4 hr incubation (Table 1). In present study, P-GO-TiO_2 NC is biocompatible at 50, 25, 10 µg/ml doses in caprine erythrocytes and similar results reported in human erythrocytes incubated with TiO_2 nanoparticle (Ghosh *et al*., 2013), < 1% (Kim and Shin, 2014) and 4.7 % (Laloly *et al*., 2014) hemolysis under exposure of 100 µg/ml Ag nanoparticle. However, 20 nmol/L and above doses of acid oxidized SWCNTs inhibited erythrocytes lysis (Donkor *et al*., 2009) but, Ag nanoparticles lysed 50% erythrocytes at 700 µg/ml doses and above (Choi *et al*., 2011).

CONCLUSION

Caprine WJ-MSCs isolated in this study were fibroblast-like cells and P-GO-TiO_2 NC have severe cytotoxic effects on caprine WJ-MSCs when they were exposed to higher doses (100 and 50 µg/ml), however, P-GO-TiO_2 NC in lower doses (25 and 10 µg/ml) are biocompatible and stimulate the caprine WJ-MSCs growth *in vitro*.

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