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Original Article

IN VITRO CYTOTOXICITY ASSESSMENT OF GRAPHENE QUANTUM DOTS IN CAPRINE WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS

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Abstract: ***Present study was designed to investigate the cytotoxicity of graphene quantum dots (GQD) in caprine Wharton's jelly derived mesenchymal stem cells (WJ-MSCs). In vitro caprine WJ-MSCs were isolated, cultured up to 3rd passage and characterized by alkaline phosphatase staining. Cytotoxicity of GQD nanoparticle was evaluated by observing alterations in cell morphology, cell viability, growth kinetics, population doubling time, colony forming unit (CFU) assay and MTT assay in different treatment groups (low dose: 10 µg/ml, moderate dose: 50 µg/ml and high dose: 100 µg/ml of GQD concentrations) in comparison with control group (without GQD). High and moderate dose of GQD exposed cells showed morphological changes and cell viability assay demonstrated a highly significant (P<0.01) decrease in the number of viable cells. Declined growth rate and highly significant (P<0.01) increase in population doubling time (PDT) of high and moderate dose treated cells were observed as compared to control group and low dose treatment group. The results of the CFU assay revealed that the cells exposed to high and moderate dose of GQD showed declined number of clones than control group and the low dose treated cells. The absorbance values of MTT assay showed non-significant (P>0.05) decrease in high and moderate dose treatment groups. In conclusion, results indicates that the dose of the nanoparticle is a potential contributing factor in exhibiting toxicity, as higher and moderate dose (100 µg/ml & 50 µg/ml) are more toxic than lower dose (10 µg/ml) in caprine WJ-MSCs.***

Key words: Graphene, Mesenchymal stem cells, Caprine

INTRODUCTION

Nanotechnology is manipulation of matter at nanoscale and applying their unique size dependent properties in various fields like drug delivery and discovery, disease diagnostics, electronics, foods, and cosmetics [1]. Recently, progress has been made by using these materials in medical applications and stem cell research [2]. In biological sciences, stem cell nanotechnology has emerged as an exciting field, where focus is on applications of nanotechnology in stem cell research. Stem cells can self-renew and differentiate into diverse cell types; broadly they are classified as embryonic stem cells and adult stem cells. Wharton's jelly is the gelatinous connective tissue from umbilical cord can serve as a good source of mesenchymal stem cells (MSCs) and have potential role in treatment of liver diseases, cardiac diseases, nerve repair, cardiac and cartilage regeneration [3,4].

Nanotechniques are applied in stem cell research, recently magnetic and fluorescent nanoparticles were used for rapid and specific isolation and sorting stem cells. Similarly, nanomaterials were also used to modify the micro-environment or three dimensional (3D) niches of stem cells for their long term maintenance and differentiation into specific cell lineages [5]. Nanoparticles were also used in stem cell or tissue engineering to design scaffolds for organ or tissue regeneration [6]. Nanomaterials were used for efficient stem cell transfection and as molecular detectors and biosensors to perceive specific molecules in the stem cell pathway. Stem cell tracking and imaging with nanomaterial will enhance the precision of the *in vivo* experiments [7].

However, prior the appliance of nanoparticles in stem cell research their biosafety needs to be assessed, many researchers reported toxicity of different nanoparticles. *In vitro* different cell lines were used to study cytotoxic effect of different nanoparticles such as molybdenum, Fe3O4, TiO2 and aluminium nanoparticles in rat liver-derived cell line (BRL 3A) [8], iron oxide (Fe_3O_4) in primary mouse connective tissue cells (L929 fibroblast) [9], titania nanoparticles and carbon nanotubes in (A549) lung epithelial cell line [10], graphene oxide in human bone marrow neuroblastoma cell line (SK-N-SH) and human epithelial carcinoma cell line (HeLa) [11], carbon encapsulated iron nanoparticles in human melanoma (HTB-140), mouse melanoma (B16-F10) cells, and human dermal fibroblasts [12], gold nanocages in human mesenchymal stem cells [13].

Many studies have been performed on the biological effects and cytotoxicity of the carbon based nanoparticles such as multi-walled carbon nanotubes [14-17], fluorescent carbon dots [18], single-walled carbon nanotubes [15,19], graphene layers [20], carbon nanotubes [10], graphene, nanodiamonds, carbon dots, and carbon black [15], graphene oxide [11] carbon encapsulated iron nanoparticles [12], fluorescent carbon nanoparticles [21]. However, there are limited reports on the cytotoxicity studies in stem cells [22-25] and the efficient implication of nanoparticles in stem cells needs their toxicity assessment. Present study was designed to isolate culture and characterize caprine Wharton's jelly derived mesenchymal stem cells and to investigate the *in vitro* cytotoxicity of graphene quantum dots in caprine WJ-MSCs.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Himedia (India) unless otherwise indicated. Graphene quantum dots were generously provided by Department of Zoology, Banaras Hindu University (UP, India).

Experimental design: *In vitro* cytotoxicity of different concentrations of GQD in caprine WJ-MSCs were assessed by designing four groups. Caprine WJ-MSCs treated with three different concentrations i.e. 10µg/ml, 50µg/ml and 100µg/ml of GQD in Dulbecco's modified Eagle's media (DMEM) whereas DMEM without GQD was maintained as control group.

Isolation and culture of caprine wharton's jelly derived mesenchymal stem cells: Uteri of pregnant (~45 days) goats (n = 5) were collected from the local abattoir and transported within 2 hours. The umbilical cords were processed for isolation of WJ-MSCs as previously described by [26], with some modifications. Briefly, Wharton's jelly was isolated from umbilical cord and washed thrice with PBS by centrifugation at 1000 rpm for 10 minutes. Finally, it was washed in serum supplemented DMEM and processed for culture. Wharton's jelly explants were gently suspended and plated in 35 mm culture dish with DMEM medium supplemented with 15% FBS (Sigma, USA), 200 IU/ml penicillin, and 200 µg/ml streptomycin at 37 ^{0}C in 5% CO_2. Explant culture was observed for attachment at 48 hr, media was replaced every third day and after 5-6 days explants were removed. After two weeks, cells attend conûuence and they were harvested using accutase and reseeded in culture plate. All the cytotoxicity assays were performed on WJ-MSCs of 3rd passage.

Alkaline phosphatase staining: Caprine WJ-MSCs were characterized by alkaline phosphatase (AP) staining as previously described by [27]. Briefly, media was removed from culture plates, cells were rinsed with Dulbecco's phosphate buffered saline (DPBS) and fixed in 4% paraformaldehyde for 10 minutes, fixed cells were again washed with DPBS and incubated in AP staining solution containing 25 mM Tris-HCl, 150 mM NaCl, 8 mM $MgCl_2$, 0.4 mg/ml Naphthol AS-MX Phosphate and 1mg/ml Fast Red TR salt for 1 hour at 37°C.

Cytotoxicity Assays

Cell morphology: Confluent monolayers of third passage caprine WJ-MSCs were treated with different concentrations of GQD for 48 hrs. The morphological alterations were observed by Nikon Diaphot 300 microscope in treatment and DMEM control group.

Cell viability: Caprine WJ-MSCs were seeded at density of $5x10^5$ cells/ml and treated with different concentrations of GQD in triplicates. Cell viability was assessed 24 hr and 48 hr post-exposure by the standard procedure for trypan blue dye exclusion technique described by [28].

Cell growth kinetics: The growth characteristics of WJ-MSCs were evaluated at various GQD concentrations in culture media. WJ-MSCs of third passage were seeded in 24 well plate at the rate of 10,000 cells per well for each treatment, respective media was changed every 72 hours. At every 48 hour, 2 wells were harvested per treatment and cell number was counted using hemocytometer for different GQD concentrations and growth curves were plotted for different treatment groups and compared with control group.

Population doubling time: Third passage WJ-MSCs were plated into a 24-well plate at 10,000 cells/well with different GQD concentrations in media. WJ-MSCs were then harvested with accutase and the total number of cells in each culture well was counted using a hemocytometer at 24 hours interval for 3 consecutive days. The population doubling time (PDT) was calculated using the equation, PDT= Culture time (CT)/Cell doubling (CD) where CD=log (N_H/N_I)/ log 2, N_H is harvested cell number and N_I is initial cell number.

Colony forming unit (CFU) assay: Clonogenic property of third passage WJ-MSCs was evaluated at different GQD concentrations, total 100 cells were seeded per tissue culture dish (96 X 16mm) and respective media refreshed twice weekly. Enumeration of colonies was done on day 14, plates were rinsed with PBS and cells were formalin fixed. Crystal violet (0.5%) staining was performed and aggregates of more than 50 cells were considered as clone, numbers of clones were counted for different treatments and control group.

MTT assay: Colorimetric assay was performed to study cell proliferation and cytotoxicity of different concentrations of GQD. WJ-MSCs were cultured in 24 well culture plates and after Accutase treatment 100 µl cell suspensions were seeded in a 96 well plate with the different concentrations of GQD in DMEM. 3-[4, 5-dimethylthiazol-2-yl] - 2, 5-diphenyltetrazolium bromide (MTT) assay was performed as per manufacturer's instructions. Briefly, WJ-MSCs monolayers in all the treatment groups including control were incubated for 4 hours with MTT reagent. Culture medium was aspirated without disturbing the monolayer and solubilization solution was added equal to the culture volume. Gentle stirring was done to completely dissolve the MTT formazan crystals and absorbance was observed at 620 nm.

Statistical analysis: Each experiment of cytotoxicity assay was conducted at least in triplicates and all data reported here are expressed as mean ± standard deviation (SD) values. One-way analysis of variance (ANOVA) was applied for the data and values of $P < 0.05$ are considered to be statistically signiû-cant. All the analysis was executed using SPSS 11.

RESULTS

Isolation and culture of caprine wharton's jelly derived mesenchymal stem cells: Caprine WJ-MSCs grows at the periphery of Wharton's jelly explants by third day and exhibits fusiform or spindle shape. After removal of explants individual fibroblastoid colonies were formed from sprouting WJ-MSCs and morphologically homogeneous populations formed confluent monolayer by day 14 (Fig. 1).

Alkaline phosphatase staining: Caprine WJ-MSCs of third passage showed high alkaline phosphatase activity and cells appeared red 15 minutes post staining (Fig. 1). Unstained WJ-MSCs were maintained as control which demonstrated no variation.

Cytotoxicity Assays

Cell Morphology: After 48 hrs in culture, caprine WJ-MSCs showed no morphological changes in 10 µg/ml treatment whereas 50 µg/ml treatment exhibited slight rounding and detachment of monolayer as compared to DMEM control. However, 100 µg/ml treatment group showed significant

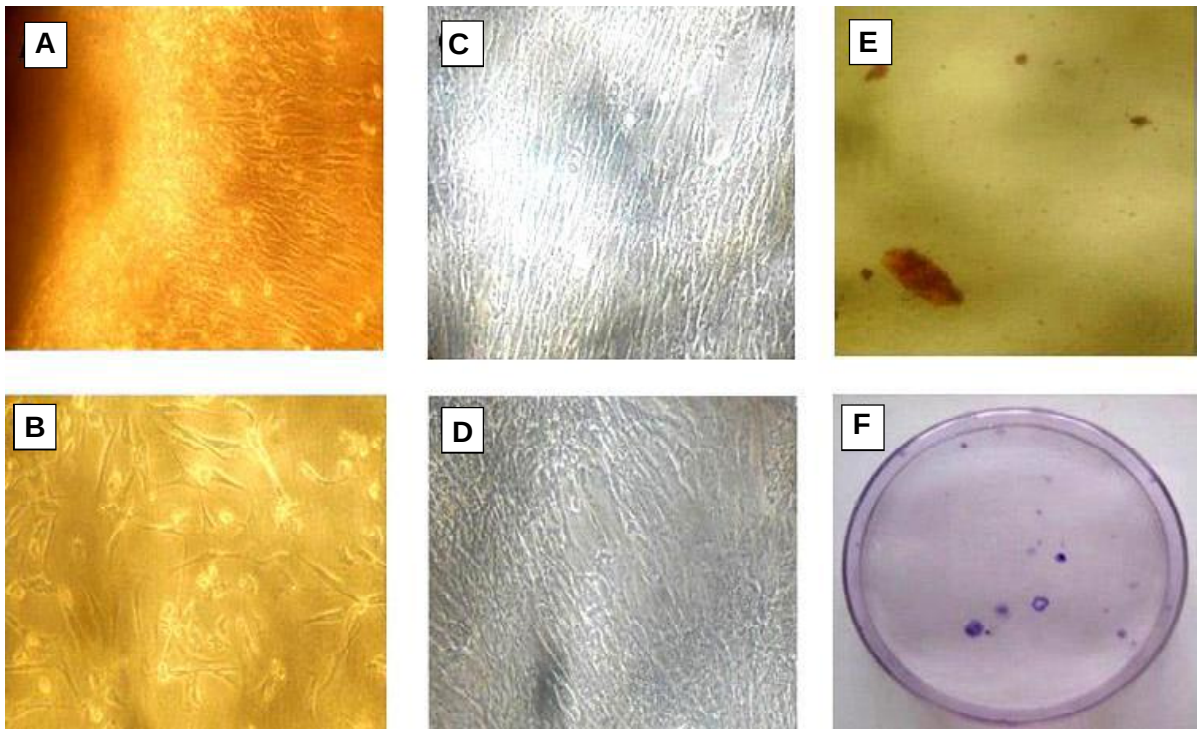


Fig. 1: Caprine WJ-MSCs day 0 (A), day 7 (B), day 14 (C), confluent monolayer (D), Alkaline Phosphatase staining of Caprine WJ-MSCs (E) and Colony forming unit assay (F)

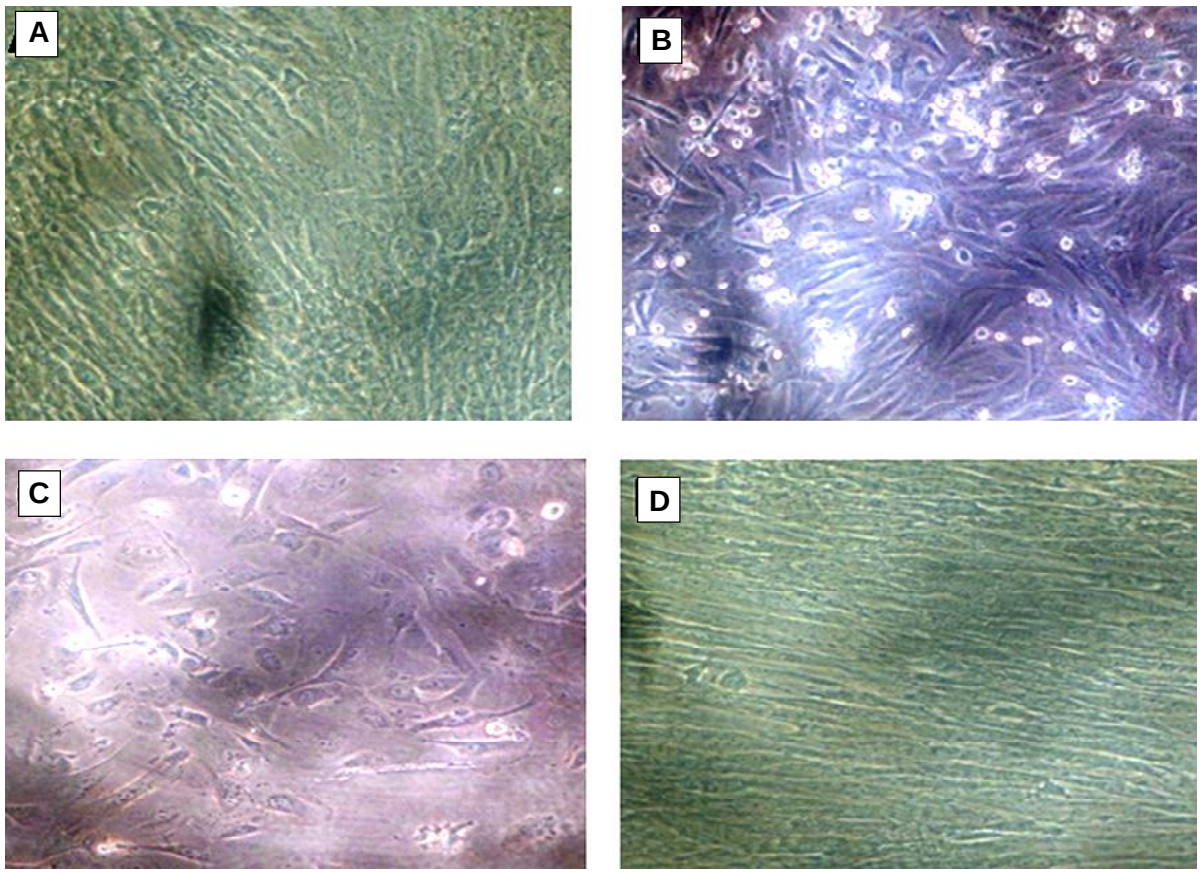
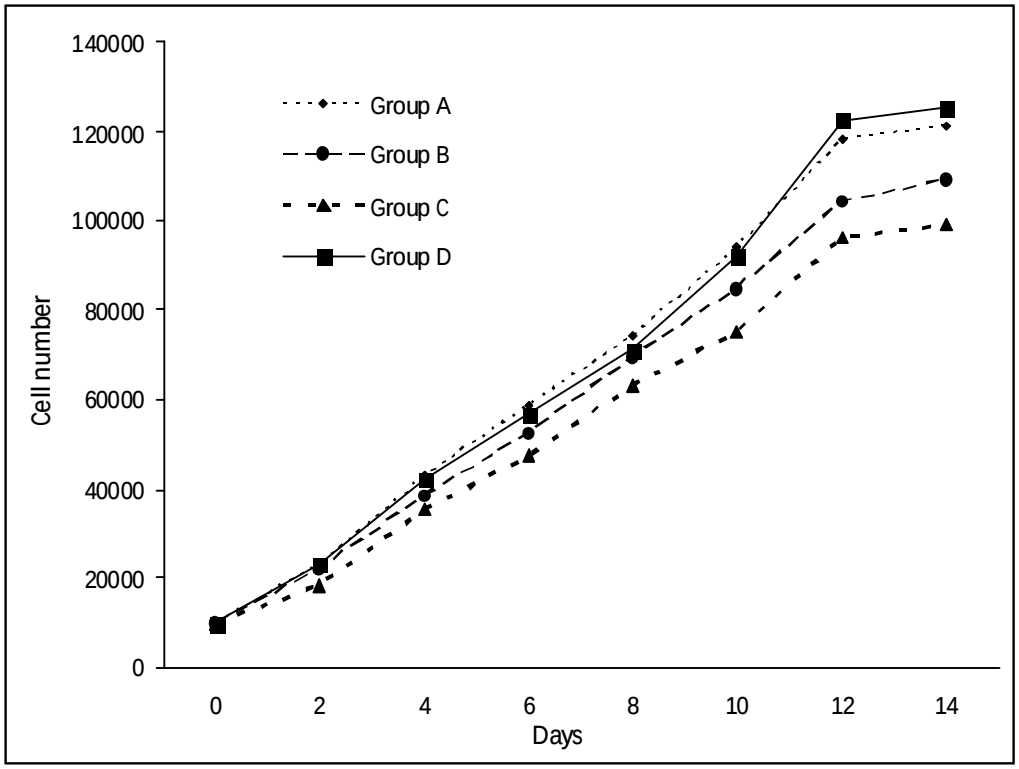


Fig. 2: Morphological changes in Caprine WJ-MSCs in different treatments (A: 10 µg/ml of GQD, B: 50 µg/ml of GQD and C: 100 µg/ml of GQD in DMEM) and control group (D: DMEM without GQD)

Fig. 3: Growth curves of treatment groups (Group A: 10 µg/ml of GQD, Group B: 50 µg/ml of GQD and Group C: 100 µg/ml of GQD in DMEM) and control group (Group D: DMEM without GQD)



morphological alterations with more number of round and detached cells (Fig. 2).

Cell viability: Cell viability assessment after 24 hr demonstrated highly significant ($P<0.01$) increase in cells exposed to 10 µg/ml as compared to DMEM control. However, highly significant ($P<0.01$) decrease in the percentage of viable cells exposed to 50 µg/ml and 100 µg/ml. Viability after 48 hours of incubation showed highly significant ($P<0.01$) decrease in the number of viable cells exposed to 50 µg/ml and 100µg/ml as compared to cells exposed to 10 µg/ml and control group (Table 1).

Cell growth kinetics: Caprine WJ-MSCs followed a normal growth curve consisting of a short initial lag phase followed by exponential growth phase with rapidly increasing cell number and ending with stationary phase. The treatment 10 µg/ml of GQD demonstrated no significant change on multiplication and *in vitro* growth of WJ-MSCs and showed displayed growth curve similar to control. However, 50 µg/ml and 100 µg/ml treatments had noticeable declined growth rate and altered shape of growth curve (Fig. 3).

Population doubling time: PDT assessment of caprine WJ-MSCs revealed cell number was doubled in 39.54±0.13 hrs and doubling time was least affected in 10µg/ml treated group (40.16 ± 0.14 hrs) and severe changes were recorded in 50µg/ml (41.90±0.15 hrs) and 100µg/ml (54.98±0.85 hrs) treatments as compared to control (Table 2).

Colony forming unit (CFU) assay: Caprine WJ-MSCs grow into individual clones indicating the existence of cell population with colony forming capability (Fig. 1). In 10 µg/ml treatment group caprine WJ-MSCs showed same number of clones as that of control (63 clones). However, colony forming capability was severely affected in 50 µg/ml (45 clones) and 100 µg/ml (32 clones) treatment groups.

MTT assay: Mean absorbance values were measured following MTT assay at different concentrations (10, 50, and 100 ìg/ml) to caprine WJ-MSCs monolayers. The 10µg/ml treatment group (0.083 ± 0.008) showed equivalent absorbance values compared to control (0.083 ± 0.005). Results indicate there was non-significant decrease ($P>0.05$) in the

Table 1: Effect of carbon based nanoparticle on cell viability of different groups at different time intervals (Mean ± S.E) (n=3). Values bearing superscripts in column differ significantly from each other. **P<0.01 (highly significant), *P<0.05 (significant).

Treatments	Viable Cells (%)	
	24 hrs Post-exposure	48 hrs Post-exposure
Group A (10µg/ml of GQD)	$90.51^{a**} \pm 0.22$	$83.79^{a**} \pm 0.13$
Group B (50µg/ml of GQD)	$80.52^{c**} \pm 0.23$	$75.53^{b**} \pm 0.16$
Group C (100µg/ml of GQD)	$74.22^{d**} \pm 0.52$	$71.04^{c**} \pm 0.57$
Group D (without GQD)	$85.71^{b} \pm 0.19$	$83.01^{a} \pm 0.23$

Table 2: Effect of carbon based nanoparticle on population doubling time (PDT) of WJ-MSCs of different groups (Mean ± S.E) (n=3). Values bearing superscripts in column differ significantly from each other. **P<0.01 (highly significant), *P<0.05 (significant).

Group	PDT (Hrs)
Group A (10µg/ml of GQD)	$40.16^{bc*} \pm 0.14$
Group B (50µg/ml of GQD)	$41.90^{b*} \pm 0.15$
Group C (100µg/ml of GQD)	$54.98^{a**} \pm 0.85$
Group D (without GQD)	$39.54^{c} \pm 0.13$

absorbance values in presence of 50 µg/ml (0.070±0.011) and 100µg/ml (0.066±.004) treatments.

DISCUSSION

Present study demonstrated WJ-MSCs isolated from the caprine umbilical cord Wharton's jelly explants possesses fibroblastoid structure that is in accordance with earlier reports [29]. Caprine WJ-MSCs showed similar morphological characteristics like MSCs isolated from bone marrow [30] and amniotic fluid in goats [31]. WJ-MSCs were characterized by alkaline phosphatase staining as reported earlier in caprine, sheep, buffalo and pig MSCs [27,29,32,33].

Cytotoxicity evaluation of different concentration (10, 50 and 100µg/ml) of GQD was demonstrated in caprine WJ-MSCs. Moderate (50µg/ml) and high (100µg/ml) doses of GQD resulted in severe morphological changes. Similar observations were reported in human bone marrow neuroblastoma cell line and epithelial carcinoma cell line exposed to 50ìg/ml concentration graphene layers [20]. Exposure to higher doses (50 and 100µg/ml) of SWCNTs and refined nanotubes (NTs) affected morphology of human dermal fibroblast [34]. However, nanometer-sized diamonds in concentration of 25-100µg/ml were non-toxic with least changes in alveolar macrophages and neuroblastoma cells [35]. Lower doses (10µg/ml) of GQD increased live cells may be due to increased metabolic rate. Similar results were reported in human adipose derived stem cell (hADSC) treated with CNTs at lower dose (0.1 and 1 ìg/ml). Higher dose (50, and 100 ìg/ml) of CNTs reduced cell proliferation and viability significantly on day 7 [24].

Severe effects on cell viability were observed at moderate and higher concentrations of GQD. Previously MWCNTs (0.1mg/ml) exposed A549 lung epithelial cell line exhibited drastic changes in cell viability [10]. Presence of CNTs inside cells alters their morphology and proliferation and higher concentrations (80µg/ml) affect cell viability [17]. Exposure of human kidney cells to SWCNTs (0.78-200µg/ml) inhibits cell growth, induces cell apoptosis and decreased cellular adhesion ability [36]. However, exposure to fluorescent carbon dots (0-200 µg/ml) not altered the viability and morphology in human breast cancer and colorectal adenocarcinoma [18].

Caprine WJ-MSCs have similar growth pattern and population doubling time as compared to previous reports in goat [37], buffalo [27], ovine [32] and pig [33] MSCs. Growth rate and doubling time of caprine WJ-MSCs is directly dependent on concentrations of GQD. Declined growth rate and highly significant increase in population doubling time of cells was similar to previous studies [38]. SWCNTs (25 and 50 µg/ml) cause growth inhibition and significant decline in growth rate of treated cells [38]. However, growth pattern in mouse cell line (L929) and human gingival fibroblasts exposed to NDs (400 mg/ml) was not affected [39]. Clonogenic capability was compromised in high and moderate concentrations of GQD and result coincides with reports of MWCNTs (100 µg/ml) exposed lung cells [40] and Sb_2O_3 exposed human hematopoietic progenitor cells [28].

The absorbance values of MTT assay indicates the mitochondrial dehydrogenase activities in the living cells and lower values in treatment groups reveals the cytotoxicity of GQD (50 and 100 µg/ml). SWCNTs (11.30 µg/cm^2) and MWCNTs (3.06 µg/cm^2) were toxic whereas carbon fullerenes (226 µg/cm^2) were non-toxic to alveolar macrophages [41]. Fluorescent carbon nanoparticles are toxic at higher doses (< 0.20 mg/ml) in HeLa cell line [21]. Al, MoO_3, Fe_3O_4 and TiO_2 nanoparticles are toxic at higher doses (250 µg/ml) and at lower doses (<50µg/ml) MoO_3 is moderately toxic, Fe_3O_4 and TiO_2 are nontoxic and Ag nanoparticles (10 µg/ml) are toxic

to rat liver cells [8]. Molybdenum nanoparticles have least effects at low dose (40 µg/ml) and at higher dose (>50 µg/ml) significant toxic to mouse spermatogonial stem cells [22]. Ag nanoparticles exhibit dose (100 µg/ml) dependent toxicity and have significant effect on mitochondrial function and membrane integrity in alveolar macrophages [42].

CONCLUSION

The key confront in application of nanomaterials is vigilant cytotoxicity assessment and detection of biocompatible doses. Results conclude that caprine WJ-MSCs can be used as potential tool to study the cytotoxicity of nanomaterials and indicate that low doses of GQD have least effects on cell growth parameters however moderate and severe effects with increasing doses. Critical data regarding tolerable concentrations of GQD in caprine WJ-MSCs can be useful for future research and applications in area of stem cell biology.

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