Journal of Animal Research: v.5 n.3, p. 415-421. September 2015

DOI: 10.5958/2277-940X.2015.00072.8

Evaluation of Dose-Dependent Cytotoxic Effects of Graphene Oxide-Iron Oxide Nanocomposite on Caprine Wharton's Jelly Derived Mesenchymal Stem Cells

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Received: 25 May, 2015 **Accepted:** 29 July, 2015

ABSTRACT

Present experiment was aimed to study the cytotoxic effects of Graphene oxide-iron oxide nanocomposite on caprine Wharton's jelly derived mesenchymal stem cells (WJ-MSCs). *Ex vivo* caprine WJ-MSCs were isolated and cultured. Cytotoxic effects of different concentrations of GO-Fe_2O_3 nanocomposite (10 µg/ml, 50 µg/ml and 100 µg/ml) were analyzed by observing cell morphology, cell viability, growth kinetics, population doubling time and colony forming unit (CFU) assay in caprine WJ-MSCs. Morphological alterations in nanocomposite-treated cells (50 µg/ml and 100 µg/ml GO-Fe_2O_3) were distinct as compared to lower dose (10 µg/ml GO- Fe_2O_3) and control group. Cell viability assay indicated a highly significant ($P<0.01$) decrease in live cell number when they were exposed to 100 µg/ml and 50 µg/ml GO- Fe_2O_3 nanocomposite and these effects were intensified with time (24 h & 48 h post-exposure). Retarded growth rate and significant ($P<0.01$) increase in population doubling time (PDT) of exposed cells (50 µg/ml and 100 µg/ml) were observed as compared to control group and low dose treatment group (10 µg/ml). Colony forming unit (CFU) assay indicated that cells treated with 50 µg/ml and 100 µg/ml nanocomposite formed less number of clones than control group and 10 µg/ml treatment group. On the basis of results, we conclude that lower doses (10 µg/ml) of the nanocomposite are safer in caprine WJ-MSCs however with increasing doses of nanocomposite (50 µg/ml & 100 µg/ml) the potential toxicity increases. Present study reports the tolerable doses of GO-Fe_2O_3 nanocomposite which will help in future applications like tracking, imaging and differentiation of caprine WJ-MSCs.

Keywords: Stem cells, nanocomposite, graphene oxide, iron oxide, nanotoxicity

In recent years, nanotechnology is applied in medical and biological sciences for multiple applications like drug development and delivery, disease diagnostics and therapeutics. Stem cells research is another novel field in regenerative medicine and nanomaterials can be employed in this field (Clarkson *et al.,* 2004). Stem cells have capability of self-renewal and multi-lineage differentiation potential. Wharton's jelly from umbilical cord is potentially good source of mesenchymal stem cells (MSCs) (Batsali *et al.,* 2013). Varieties of nanomaterials are studied for isolation and sorting of stem cells, micro-environment or three dimensional (3D) culture systems for tissue engineering (Chen *et al.,* 2013). It is also applied for stem cell transfection and as molecular detectors and biosensor. Stem cell tracking and imaging with nanomaterial is another crucial factor in regenerative medicine (Kaur and Singhal, 2012). Recently, stem cell nanotechnology research has emerged as a new exciting field, where focus is on application of nanoparticles in stem cell biology. Identification of rapid, easy and efficient method for stem cell isolation and their long term maintenance is major challenge in stem cell research. Differentiation of stem cells into specific cell lineages and precise mimicking of microenvironment of stem cells are also key confront. The development of novel regenerative therapies for chronic, debilitating and various unresponsive clinical diseases

and disorders of animals as well as humans is also crucial (Ribitsch *et al.*, 2010).

One of the important criteria for use of nanoparticles is their assessment for cellular toxicity. Many *in vitro* experimentation reports indicate the cytotoxic effects of nanoparticles on human epithelial carcinoma cell line (HeLa), lung epithelial cells and dermal fibrblasts (Chen *et al.*, 2012, Ireneusz *et al.*, 2013, Wadhwa *et al.*, 2011). Some researchers have also assessed effects of carbon nanotubes, silver nanoparticles, iron oxide and gold nanocages on stem cells (Braydich-Stolle *et al.*, 2005; Ahamed *et al.*, 2008; Esfandiary *et al.*, 2014; Fan *et al.*, 2013, Yu *et al.*,2013). The efficient implication of nanoparticles in stem cells need their toxicity assessment therefore present experiment was designed to study *in vitro* dose dependent cytotoxicity of graphene oxide iron oxide nanocomposite in caprine WJ-MSCs.

MATERIALS AND METHODS

All Chemicals and reagents were purchased from Himedia (India) unless otherwise mentioned. Graphene oxide iron oxide nanocomposite material was kindly supplied by Department of Zoology, Banaras Hindu University (UP, India).

Isolation and Culture of Caprine Wharton's Jelly derived Mesenchymal Stem Cells

Gravid uteri (~45 days) goats (n = 6) were collected from the local abattoir and transported rapidly to laboratory. Umbilical cords were separated and processed for isolation of Wharton's jelly as per protocol followed by Babaei and coworkers (2008) with partial modifications. Wharton's jellies were separated and washed with Dulbecco's phosphate buffer saline (DPBS) and further processed by centrifugation and washing with serum supplemented DMEM. Explant culture was maintained in culture plate (35 mm) with DMEM medium supplemented with 15% FBS (Sigma, USA), 200 IU/ml penicillin, and 200 µg/ml streptomycin at 37 °C in 5% CO_2. Attachment of WJ-MSCs was observed at 48 h , culture media was replaced every third day and cells were maintained at 37 °C in 5% CO_2 and after 5-6 days explants were removed. Once confluent monolayers were formed WJ-MSCs were detached using accutase and reseeded in culture plate. Caprine WJ-MSCs were maintained up to third passage in DMEM medium supplemented with 15% FBS (Sigma, USA), 200 IU/ml penicillin, and 200 µg/ml streptomycin at 37 °C in 5% CO_2.

Cytotoxicity Assays

Caprine WJ-MSCs of third passage were treated with low dose (10 µg/ml), moderate dose (50 µg/ml) and high dose (100 µg/ml) of GO-Fe_2O_3 nanocomposite in Dulbecco's modified Eagle's media (DMEM) whereas control group of caprine WJ-MSCs was maintained in DMEM without GO-Fe_2O_3 nanocomposite. All cytotoxicity assays were performed in triplicate and duration of exposure was variable in different parameters. Cell morphology was observed after 48 h of exposure and cell viability after 24 h and 48 h after exposure. Population doubling time was calculated during exposure of 72 h whereas cell growth kinetics and colony forming unit assay was studied during 14 days of exposure to nanocomposite. All treated and untreated cells were maintained at 37°C in 5% CO_2.

Cell Morphology

The morphological changes in different treatments (10 µg/ml, 50 µg/ml and 100 µg/ml) and control caprine MSCs were observed by using Nikon Diaphot 300 microscope. Any alteration in shape and size of fibroblastoid caprine WJ-MSCs was recorded.

Cell Viability

Caprine WJ-MSCs were plated at density of $5x10^5$ cells/ml in tissue culture flask (25 cm^2) and treated with various concentrations of GO-Fe_2O_3 nanocomposites in triplicates. Cell viability was observed 24 h and 48 h post-exposure using trypan blue dye exclusion technique as described by Bregoli and coworkers (2009).

Cell Growth Kinetics

Caprine WJ-MSCs were exposed to GO-Fe_2O_3 nanocomposite and cellular growth kinetics was evaluated during exposure period of 14 days. Caprine WJ-MSCs of third passage were cultured at the rate of 10,000 cells per well for each test group (10 µg/ml, 50 µg/ml and 100 µg/ml) and respective media was changed every 72 h. At every 48 h, 2 wells were harvested per treatment and cell

number was counted using hemocytometer for different treatments and control. Growth curves were plotted for different treatment groups and compared with control group.

Population Doubling Time

Caprine WJ-MSCs were seeded at rate of 10,000 cells/well with different GO-Fe_2O_3 nanocomposite concentrations in serum supplemented DMEM. Total number of cells in each culture well of 24-well plate was counted using a hemocytometer at 24 h interval for 3 consecutive days. 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

Total 100 cells were seeded per culture plate (96X16mm) and respective media containing 10 µg/ml, 50 µg/ml and 100 µg/ml of GO-Fe_2O_3 nanocomposite was changed twice weekly. Clonogenic ability of WJ-MSCs was evaluated and numbers of colonies were counted on day 14. Plates were rinsed with PBS followed by formalin fixation and crystal violet (0.5%) staining. Clusters of more than 50 cells were considered as clone, numbers of clones were counted for different treatments and control group.

Statistical Analysis

All experiments were conducted in triplicate and data is expressed as mean± standard deviation. One way analysis of variance (ANOVA) was applied using SPSS 11 and values of means at $P< 0.05$ are considered significant whereas as highly significant at $P< 0.01$.

RESULTS AND DISCUSSION

In present study, caprine Wharton's jelly explants were isolated, cultured and by third day WJ-MSCs appears on periphery of explants. Distinct fibroblastoid cells were forming colonies of WJ-MSCs and morphologically homogenous cells formed confluent monolayer by day 14 (Figure 1). Similar morphological observations were reported in caprine WJ-MSCs by previous researchers (Moshrefi *et al.*, 2010, Dar *et al.*, 2015). Caprine WJ-MSCs showed similar morphological characteristics like MSCs isolated from bone marrow (Baghaban *et al.*, 2009) and amniotic fluid in goats (Pratheesh *et al.*, 2013).

Figure 1: Caprine Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) at the periphery of explant at Day 3 (A: 20X), Fibroblast like cells on Day 7 (B: 20X), Confluent monolayer (C: 20X)

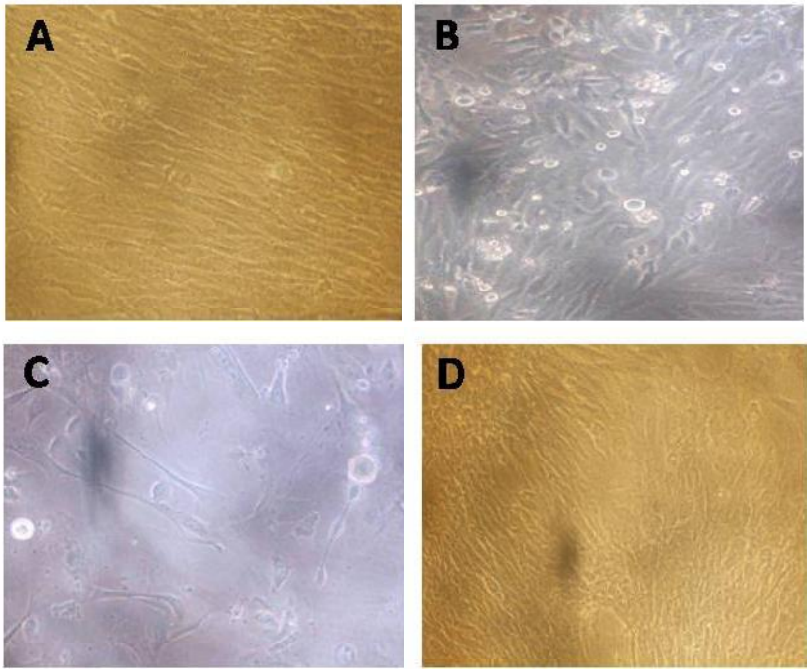

Figure 2: Fibroblast like Caprine WJ-MSCs in 10 µg/ml of GO-Fe_2O_3 nanocomposite (A:20X) and control group without GO-Fe_2O_3 nanocomposite exposure (D:20X); Moderate number of detached, round floating dead cells in 50 µg/ml treatment (B:20X) and Severe detachment, floating dead and elongated WJ-MSCs in 100 µg/ml treatement (C:20X)

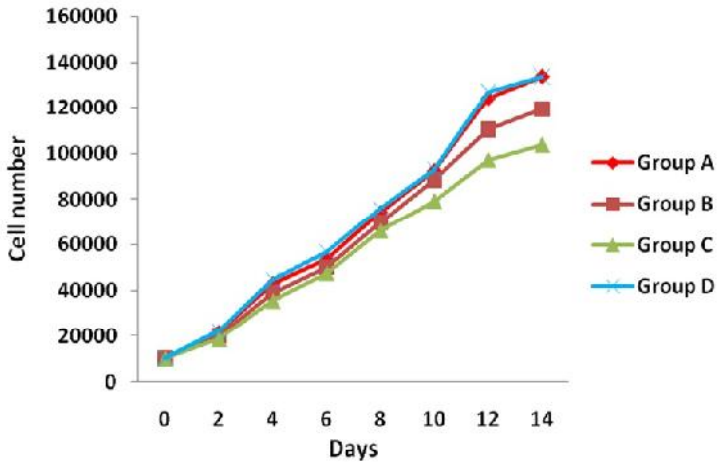

Figure 3: Comparison of growth curves of treatment groups (A: 10 µg/ml of GO-Fe_2O_3 nanocomposite, B: 50 µg/ml of GO-Fe_2O_3 nanocomposite and C: 100 µg/ml of GO-Fe_2O_3 nanocomposite in DMEM) and control group (D: DMEM without GO-Fe_2O_3 nanocomposite)

Morphological observations of caprine WJ-MSCs revealed that lower dose treatment group had least alterations than control group however distinct phenotypic changes were seen in moderate dose treatment group. Detachment of cells and

more number of round floating dead cells were observed in high dose treatment group (Figure 2). Moderate (50 µg/ml) and high (100 µg/ml) doses of GO-Fe_2O_3 nanocomposite resulted in severe morphological changes. Earlier study reported similar changes in caprine MSCs exposed to graphene oxide nanoparticle and effects were dose dependant (Dar *et al.*, 2015). Human bone marrow neuroblastoma and epithelial carcinoma cells treated with graphene layers (50 µg/ml) exhibited similar changes in morphology (Zhang *et al.*, 2010). Dose-dependent changes were reported in morphology of human dermal fibroblast exposure to higher doses (50 and 100 µg/ml) of SWCNTs and refined nanotubes (NTs) (Tian *et al.*, 2006).

Caprine WJ-MSCs after 24 h exposure to 50 µg/ml and 100 µg/ml of GO-Fe_2O_3 nanocomposite had highly significant (P<0.01) decrease in live cell number as compared to WJ-MSCs exposed to 10 µg/ml and control. Similar trend was followed after 48 hours of incubation and showed highly significant (P<0.01) decrease in the number of viable cells exposed to 50 µg/ml and 100 **µg/ml as compared to cells exposed to 10 µg/ml and control group (Table 1).**

Table 1. Effect of graphene oxide iron oxide nanocomposite on cell viability of different treatment groups at different time intervals (Mean ± S.E) (n=3)

Treatments	Viable Cells (%)	
	24 hrs Post-exposure	48 hrs Post-exposure
Group A (10µg/ml of GO-Fe_2O_3)	88.97^{a*} ± 1.61	81^{a*} ± 0.47
Group B (50µg/ml of GO-Fe_2O_3)	79.5^{b**} ± 0.71	75.33^{b**} ± 0.72
Group C (100µg/ml of GO-Fe_2O_3)	73.83^{c***} ± 0. 76	68.83^{c***} ± 0.72
Group D (without GO-Fe_2O_3)	86.89^{a*} ± 0.26	82.5^{a*} ± 0.62

Values bearing superscripts in column differ significantly from each other. ***P<0.01 (highly significant), ^{a,b,c} P<0.05 (significant).

Previous experiments reported significant increase in viable cell number of caprine WJ-MSCs and human adipose derived stem cells exposed to lower dose of GO quantum dots (Dar *et al.*, 2015) and carbon nanotubes (Esfandiary *et al.*, 2014) respectively. However same reports depict increased cell death after exposure to moderate and higher doses. Similar results were reported in A549 lung epithelial cell line (Wadhwa *et al.*, 2011) and human kidney cells (Cui *et al.*, 2005) exposed to various concentrations of CNTs.

Table 2. Effect of carbon based nanoparticle on population doubling time (PDT) of WJ-MSCs of different groups (Mean ± S.E) (n=3)

Group	PDT (Hrs)
Group A (10µg/ml of GO-Fe_2O_3)	43.05^{c**} ± 0.47
Group B (50µg/ml of GO-Fe_2O_3)	48.75^{b**} ± 1.27
Group C (100µg/ml of GO-Fe_2O_3)	54.26^{a***} ± 1.47
Group D (without GO-Fe_2O_3)	41.95^{c*} ± 0.34

Values bearing superscripts in column differ significantly from each other. ***P<0.01 (highly significant), ^{a,b,c} P<0.05 (significant).

Normal growth curve of caprine WJ-MSCs was plotted and compared with treatment groups. Growth curve exhibits initial lag phase followed by log phase of rapid growth and platue of stationary phase. Caprine WJ-MSCs have similar growth pattern and population doubling time as reported previously in caprine (Dar *et al.*, 2015), bovine (Gade *et al.*, 2013) and ovine (McCarty *et al.*, 2009). Lower dose treatment group had similar growth curve as observed in control group but significant modifications were pointed in moderate and high treatment groups. Changes in growth curve suggest declined growth rate and altered shape of growth curve (Figure 3). Results are in accordance with our previous experiment in GO quantum dots in caprine MSCs (Dar *et al.*, 2015) and indicate impact of nanocomposite on growth rate of caprine MSCs. SWCNTs (25 and 50 µg/ml) inhibited growth and significant decline in growth rate was observed in exposed cells (Olga *et al.*, 2008). PDT of caprine WJ-MSCs severely affected due to reduced growth of nanocomposite exposed caprine MSCs and this effect was dose dependant. In control group cell number was doubled in 41.95**±0.34** h and doubling time was least affected in low dose treatment (43.05 ±0.47 h) and severe alteration was observed in moderate dose (48.75±1.27 h) and high dose (54.26±1.47 h) treatments (Table 2). Highly significant increase in population doubling time of cells was similar to previous studies (Dar *et al.*, 2015 and Olga *et al.*, 2008). Clonogenic property of caprine WJ-MSCs expressed by growth of individual clones from

WJ-MSCs and confirms their colony forming capability. Low dose treatment group showed nearly similar number of clones as that of control group (64 clones) but colony forming capability was severely affected in moderate dose (48 clones) and high dose (36 clones) treatment groups. Similar effect on CFU was observed in GO quantum dots exposed caprine MSCs (Dar *et al.*, 2015), MWCNTs (100 µg/ml) exposed lung cells (Asakura *et al.*, 2010) and Sb_2O_3 exposed human hematopoietic progenitor cells (Bregoli *et al.*, 2009).

Results conclude that lower dose of GO-Fe_2O_3 nanocomposite have least impact on cellular morphology, viability, clonigenic property and growth kinetics of caprine WJ-MSCs however increasing concentration of nanocomposite have more severe negative impact on these parameters.

Acknowledgement

The authors are highly thankful to the Dean, College of Veterinary Science and Animal Husbandry, Chhattisgarh Kamdhenu Vishwa Vidyalaya, Anjora, Durg (CG) for providing necessary facilities to carry out this research work.

REFERENCES

Ahamed, M., Karns, M., Goodson, M., Rowe, J., Hussain, S.M., Schlager, J.J. and Hong, Y. 2008. DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. *Toxicol. Appl. Pharmacol*., **233** (3):404–410.

Asakura, M., Sasaki, T., Sugiyama, T., Takaya, M., Koda, S., Nagano, K., Arito H. and Fukushima, S. 2010. Genotoxicity and cytotoxicity of multi-wall carbon nanotubes in cultured Chinese hamster lung cells in comparison with chrysotileA fibers. *J. Occup. Health*,**52**(3):155–166.

Babaei, H., Moshrefi, M., Golchin, M. and Nematollahi-Mahani, S.N. 2008. Assess the pluripotency of caprine umbilical cord Wharton's jelly mesenchymal cells by RT-PCR analysis of early transcription factor nanog. *Iranian J. Vet. Surg*., **3**(2):57-65.

Baghaban, M.E., Hamid, N., Fahimeh, F., Leila, T. and Mohamad, T.D. 2009. *Ex vivo* expansion and differentiation of mesenchymal stem cells from goat bone marrow. *Iran. J. Basic Med. Sci.*, **12**(2):70-79.

Batsali, A.K., Kastrinaki, M.C., Papadaki, H.A. and Pontikoglou, C. 2013. Mesenchymal stem cells derived from Wharton's Jelly of the umbilical cord: biological properties and emerging clinical applications. *Curr. Stem Cell Res. Ther.*, **8**(2):144-155.

Braydich-Stolle, L., Hussain, S., Schlager, J.J. and Hofmann, M.C. 2005. *In vitro* cytotoxicity of nanoparticles in mammalian germline stem cells. *Toxicol. Sci.*, **88**(2):412–419.

Bregoli, L., Chiarini, F., Gambarelli, A., Sighinolfi, G., Gatti, A.M., Santi, P., Martelli, A.M. and Cocco, L. 2009. Toxicity of antimony trioxide nanoparticles on human hematopoietic progenitor cells and comparison to cell lines. *Toxicology*, **262**:121-129.

Chen, H., Zeng, Y., Liu, W., Zhao, S., Wu, J.and Du, Y. 2013. Multifaceted applications of nanomaterials in cell engineering and therapy. *Biotechnol. Adv*., **31**(5):638-653.

Chen, L., Hu, P., Zhang, L., Huang, S., Luo, L. and Huang, C. 2012. Toxicity of graphene oxide and multi-walled carbon nanotubes against human cells and zebrafish. *Sci. China Chem*., **55**(10):2209-2216.

Clarkson, A.J., Buckingham, D.A., Rogers, A.J., Blackman, A.G. and Clark, C.R. 2004. Nanostructured Ceramics in Medical Devices: Applications and Prospects. *JOM*, **56**(10):38-43.

Cui, D., Tian, F., Ozkan, C.S., Wang, M.and Gao, H.2005. Effect of single wall carbon nanotubes on human HEK293 cells. *Toxicol. Lett.*, **155**:73-85.

Dar, M.R., Gade, N.E., Mishra, O.P., Khan, J.R., Kumar, V. and Patyal, A. 2015. *In vitro* cytotoxicity assessment of graphene quantum dots in caprine Wharton's jelly derived mesenchymal stem cells. *J. Cell & Tissue Res.*, **15**(1):4703-4710.

Esfandiary, E.,Valiani, A., Hashemibeni, B., Moradi, I. and Narimani, M. 2014. The evaluation of toxicity of carbon nanotubes on the human adipose-derived-stem cells in-vitro. *Adv. Biomed Res*., **3**:40.

Fan, J., Tan, Y., Jie, L., Wu, X., Yu, R. and Zhang, M. 2013. Biological activity and magnetic resonance imaging of super paramagnetic iron oxide nanoparticles-labeled adipose-derived stem cells. *Stem Cell Res. Ther*., **4**(2):44.

Gade, N.E., Pratheesh, M.D., Nath, A., Amarpal, Dubey,P.K., Sharma,B., Saikumar, G.and Sharma, G.T. 2013. Molecular and cellular characterization of buffalo bone marrow derived mesenchymal stem cells. *Reprod. Domest. Anim*., **48**(3):358-67.

Ireneusz, P.G., Michal, B., Monika, A.C., Anita, K., Magdalena, P., Andrzej, C. andAgnieszka, O. 2013. Cytotoxicity evaluation of carbon-encapsulated iron nanoparticles in melanoma cells and dermal fibroblasts. *J. Nanopart. Res.*, **15**:1835.

Kaur, S. and Singhal, B. 2012. When nano meets stem: the

impact of nanotechnology in stem cell biology. *J. Biosci. Bioeng*.,**113**(1):1-4.

McCarty, R.C., Gronthos, S., Zannettino, A.C., Foster, B.K. and Xian, C.J. 2009. Characterization and developmental potential of ovine bone marrow derived mesenchymal stem cells. *J. Cell. Physiol*., **219**(2):324-333.

Moshrefi, M., Babaei, H. and Nematollahi-Mahani, S.N. 2010. Isolation and characterization of mesenchymal cells isolated from caprine umbilical cord matrix. *Anim. Reprod*., **7**(4):367-372.

Olga, Z., Palumbo, R., Bernini, R., Zeni, L., Sarti, M. and Scarfì, M.R. 2008. Cytotoxicity investigation on cultured blood cells treated with single-wall carbon nanotubes. *Sensors*, **8**:488-499.

Pratheesh, M.D., Gade, N.E., Katiyar, A., Dubey, P.K., Sharma, B., Saikumar, G., Amarpal, S., and Sharma, G.T. 2013. Isolation, culture and characterization of caprine mesenchymal stem cells derived from amniotic fluid. *Res. Vet. Sci*., **94**(2):313-9.

Ribitsch, I., Burk, J., Delling, U., Geibler, G., Gittel, C., Julke, H. and Brehm, W. 2010. Basic science and clinical application of stem cells in Veterinary medicine. *Adv. Biochem. Eng. Biotechnol*., **123**:219-263.

Tian, F.R, Cui, D.X, Schwarz, H., Estrada, G.G. and Kobayashi, H.2006. Cytotoxicity of single-wall carbon nanotubes on human fibroblasts. *Toxicol. In Vitro*, **20**:1202-1212.

Wadhwa, S., Rea, C., O'Hare, P., Mathur, A., Roy, S.S., Dunlop, P.S.M., Byrne, J.A., Burke,G., Meenan, B. and McLaughlin, J.A. 2011. Comparative *in vitro* cytotoxicity study of carbon nanotubes and Titania nanostructures on human lung epithelial cells. *J. Hazard. Mater*, **191**:56–61.

Yu, S.Z., Wang, Y., Wang, L., Wang, Y., Cai, X., Zhang, C., Wang, L.V. and Xia, Y.2013. Labeling human mesenchymal stem cells with gold nanocages for *in vitro* and *in vivo* tracking by two-photon microscopy and photoacoustic microscopy. *Theranostics*, **3**(8):532-543.

Zhang, Y., Ali, S.F., Dervishi, E., Xu, Y., Li, Z., Casciano, D. and Biris, A.S. 2010. Cytotoxicity effects of graphene and single-wall carbon nanotubes in neural phaeochromocytoma-derived PC12 cells. *ACS Nano.*, **4**:3181–3186.