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Research Article

IN VITRO CYTOTOXICITY ANALYSIS OF NITROGEN DOPED GRAPHENE OXIDE HYDROXYAPATITE NANOCOMPOSITE (N_2-GO-HA NC) IN CAPRINE WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS (WJ-MSCs) AND BLOOD CELLS

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Abstract: Present study was designed to analyze *in vitro* cytotoxicity of different concentrations of Nitrogen dope Graphene Oxide Hydroxyapatite nanocomposites (N_2-GO-HA NC) in caprine WJ-MSCs and blood cells. Caprine WJ-MSCs were isolated cultured, characterized and propagated for evaluating nanotoxicity by studying cell morphology, cell viability, growth kinetic, PDT, MTT, hemolysis and CBC assays. Caprine WJ-MSCs after 24 hrs, 48 hrs and 72 hrs exposure with 100 and 50 µg/ml of N_2-GO-HA NC showed significant decrease in viable cell number and alterations in cell morphology whereas with 25 and 10 µg/ml of N_2-GO-HA NC no significant alteration in cell morphology but significant ($P<0.01$) increased in cell viability was observed. Similar trend was observed in cell growth kinetics study where significant ($P<0.05$) decrease in cell growth with increased PDT is estimated in 100 and 50 µg/ml however exposure to 25 and 10 µg/ml doses resulted in significant ($P<0.05$) increase in growth of caprine WJ-MSCs as compared to control group. MTT assay in caprine WJ-MSCs exposed to 100 and 50 µg/ml of N_2-GO-HA NC also revealed significant ($P<0.01$) decrease in cell number as compared to 25, 10 and 0 µg/ml doses. Hemolysis assay pointed out significant ($P<0.01$) hemolysis in caprine RBCs exposed to 100 µg/ml dose as compared with 50, 25, 10 and 0 µg/ml doses of N_2-GO-HA NC. However, RBCs, WBCs and platelets count were altered non-significantly in 100, 50, 25 and 10µg/ml doses of N_2-GO-HA NC as compared to control. The report concludes that higher doses (100 and 50 µg/ml) of N_2-GO-HA induce significant toxicity to Caprine WJ-MSCs as well as blood cells (100 µg/ml), however at lower doses (25 and 10 µg/ml) slight growth enhancing effects were observed.

Keywords: *WJ-MSCs, blood cells, caprine, nanotoxicity, stem cells*

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Introduction

In present era nanotechnology has gained tremendous attention due to its broad range of applications in fields such as textile, electronics, cosmetics, transportation, energy and information technology, environment and space research [1], food industry and agriculture [2] and biomedicine [3].In biomedical science, nanotechnology gave significant breakthroughs in the area of biotechnology [4], drug delivery [5], gene delivery [6] imaging[7], biosensors [8] proteomics and genomics [9], cancer therapy [10]and in tissue engineering [11]. Stem cell based therapies are used to treat chronic and debilitating diseases in humans as well as animals and nanostructured scaffolds, tube, rods etc. have shown immense potential towards stimulation of stem cell growth and differentiation both *In vitro* and in vivo studies [12]. Stem cells are capable to differentiate in numerous cell types and adult stem cells reside in nearly all organs. Wharton's jelly (WJ) is extraembryonic tissue and rich source of stem cells. WJ derived mesenchymal stem cells (WJ-MSCs) were used in clinical application to control type I diabetes [13], cardiac diseases [14], neurological diseases [15], liver diseases [16], bone and cartilage regeneration [17]. Stem cell nanotechnology is emerging field and nanomaterials are being explored for multiple applications in stem cell research such as tissue engineering [18], stem cell culture and differentiation [12], delivery of stem cells [19], tracking of stem cells [20] and intracellular delivery of DNA and RNA [21]. These potential areas of application have attracted researchers to evaluate the potency and biocompatibility of nanomaterials in stem cells. Carbon based nanomaterial (CBNs) based scaffolds like sheets and films are well reported for improving stem cell growth, maturation and differentiation [22 and 23]. Among other, graphene (mother of CBNs) is an atom thick carbon monolayer 2 dimensional honeycomb structure, synthesized in pure form and studied in stem cell research [24]. Several chemical derivatives of graphene for *e.g.*, graphene oxide (GO), reduced graphene oxide (rGO), graphene quantum dots (GQDs) were explored in due course of time and their potential in biomedicine were examined [25]. More recently, heteroatoms doped graphene derivatives have been reported to exploit their unique properties in doped state [26]. However, potential biological applications of these doped graphene derivatives have not been studied thoroughly specially in stem cell research. Investigation of biocompatibility of these doped graphene derivatives becomes more important before their study in tissue engineering. In our previous studies we have shown a dose dependent cytotoxic effect of GQDs, a chemical derivative of graphene [27] and graphene oxide iron oxide nanocomposite (GO-Fe_2O_3 NC) in caprine WJ-MSCs [28]. In addition, graphene based nanomaterials (GBNs) have shown their cytotoxic effect against MC3T3-E1 cells [29], rat pheochromocytoma cells [30], MSCs [31], lung epithelial cells [32]. Cytotoxicity of undoped graphene and its nanocomposites like HA-rGO powder [29, 33, 34 and 35] and HA-graphene (HA-GN) sheets [36] were studied in a dose and exposure time dependent manner.

But, biocompatibility of doped of graphene with organic or inorganic material is not investigated yet. To overcome nanomaterial cytotoxicity issue and expand the horizons of biocompatible carbon nanomaterials for stem cell research, we hypothesis that, nitrogen doped graphene-hydroxyapatite nanocomposite (N_2-GO-HA NC) may reduce toxic effect of graphene and induce cell growth. Therefore, present study was designed to evaluate *In vitro* dose dependent cytotoxicity of N_2-GO-HA NC in caprine WJ-MSCs and blood cells.

Materials and methods

All chemicals, reagents and plastic wares procured from Hi-Media (India) and N_2-GO-HA NC nanomaterial was provided by Department of Physics, Banaras Hindu University, Banaras, Uttar Pradesh (India). Present study was conducted in Stem Cell Laboratory, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Anjora, Durg, Chhattisgarh.

Experimental design

In vitro cytotoxicity of nitrogen doped graphene oxide hydroxyapatite nanocomposite (N_2-GO-HA NC) was studied at different concentrations in caprine WJ-MSCs and blood cells. N_2-GO-HANC in Dulbecco's Modified Eagles Media (DMEM) was used at 100 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml and DMEM without N_2-GO-HA NC was used as control.

Isolation, culture and characterization of WJ-MSCs and collection of blood samples

Caprine gravid uteri (~ 45 days, n=4) were collected from local abattoir and transported within 2 hrs to laboratory. Umbilical cord was incised and processed for isolation of WJ-MSCs as per the procedure described by [37] with minor alterations. Isolated umbilical cord was processed by washing thrice with Phosphate Buffer Saline (PBS). WJ mesenchymal connective tissues were separated from blood vessels in petridish. WJ was washed in PBS thrice and followed by washing in Dulbecco's Modified Eagles Media (DMEM). WJ explants were seeded in tissue culture flask (12.5cm^2) with DMEM supplemented with Fetal Bovine Serum (15%), antimycotic and antibiotic solution and incubated at 37°C with 5% CO_2. Every third day media was replaced and culture was observed and on day 6th explants were removed. On day 14, confluent monolayer was observed and cells were processed for passage. Third passage Caprine WJ-MSCs were used to analyze *In vitro* cytotoxicity of N_2-GO-HA NC. Fresh 5.0 ml blood samples were collected in ethylene diamine tetraaceteic acid (EDTA) coated tubes by jugular vein puncture from three female goats and used to study cytotoxicity.

Characterization of Caprine WJ-MSCs

Third passage caprine WJ-MSCs on fifth day were characterized by Alkaline Phosphatase (ALP) staining using ALP detection kit as per the manufacturer instructions. Briefly, Caprine WJ-MSCs rinsed with Dulbecco's Phosphate Buffer Saline (DPBS) and fixed in 4% paraformaldehyde for 2 minute and washed twice with DPBS. Fixed Caprine WJ-MSCs were incubated with ALP stain solution (Fast Red Violet: Napthol AS-BI phosphate solution: Water in ratio 2:1:1) for 15 minutes at room temperature and cells were washed with DPBS and images were recorded.

In vitro cytotoxicity assays

Cell morphology

Caprine WJ-MCSs of third passage were treated with N_2-GO-HA NC in different doses and after 24, 48 and 72 hrs of exposure morphological changes were assessed by using inverted microscope (Nikon Diaphot 300) and compared with control.

Cell viability

Cell viability was assessed by using trypan blue dye exclusion technique as reported by [38] with some modifications. Caprine WJ-MSCs were seeded at density of 5 x 10^4 cells/ml and incubated with different doses of N_2-GO-HA NC and cell viability was assessed 24, 48 and 72 hrs post-exposure.

Cell growth kinetic

Growth characteristics Caprine WJ-MSCs were analyzed as per the methods described by [27]. Briefly, caprine WJ-MSCs were seeded at density 1x 10^4 cells /well in 24 cell culture plates and media containing respective doses of N_2-GO-HA NC were changed at every fourth day. Cells were harvested every after 48 hrs intervals up to day 14 and cells were counted using hemocytometer (Neubar's chamber) and growth curves were plotted and compared with control group.

Population doubling time (PDT)

Caprine WJ-MSCs were incubated with N_2-GO-HA NC in 24 well cell culture plates at density 1x10^4 cells / well for 3 days. At every 24 hrs interval cells from each cell culture plate were harvested and cells were counted using a hemocytometer (Neubar's chamber). The population doubling time (PDT) was calculated using equation described by [28],

PDT= Culture time (CT)/Cell doubling (CD)

where, CD=log (NH/NI)/ log 2, NH is harvested cell number and NI is initial cell number

MTT assay

Colorimetric assay was performed to evaluate cytotoxicity in caprine WJ-MSCs treated with different concentrations of N_2-GO-HA NC using MTT (Tetrazolium dye 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) Assay Cell Kit as per the instructions of manufacturer. Briefly, 100 µl freshly harvested caprine WJ-MSCs were incubated with different doses of N_2-GO-HA NC in wells of 96 well cell culture plate for 24 hrs and cells were incubated with MTT reagent for 4 hrs. Media was aspirated and 100 µl solubilization solution was added and gentle stirring was done to dissolve MTT formazan crystals completely and absorbance was recorded on ELISA plate reader at 620 nm.

Hemolysis assay

Hemolysis assay was done as per the method described by [39] with some modifications. Briefly, 5.0 ml freshly collected caprine blood was centrifuged at 3000 rpm for 10 minutes at 4°C and plasma along with white buffy coat aspirated and erythrocytes were washed 3 times with DPBS. 100 µl 10th dilution erythrocytes were incubated with different doses of N_2-GO-HA NC for 4 hrs in microcentrifuge tubes at 37°C along with negative and positive controls. All tubes were centrifuged at 500 rpm for 5 minute and 100µl hemoglobin in supernatant was transferred in flat bottom 96 wells tissue culture plate and absorbance was read thrice at 492 nm in ELISA plate reader and % hemolysis was determined by using following formula:

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

Complete blood count (CBC) test

CBC test was conducted using Hematology Analyzer (Urit 2900-Vet Plus). 100µl DMEM containing different doses of N_2-GO-HA NC was added in 1.0 ml caprine blood in triplicate samples and incubated for 4 hrs at 370C in incubator. CBC test was performed to determine RBCs, WBCs and platelets count.

Statistical analysis

Experimental data reported here is expressed as mean ± standard error (SE) 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

Isolation, culture and characterization of Caprine WJ-MSCs

Caprine WJ-MSCs were observed at the periphery of WJ explants and have varying morphology from spindle or fusiform shape. Typically fibroblast like cells grew and formed colonies and their number outgrow to form confluent monolayer by 14 day [Fig-1].

Table-1 Effect of exposure of different concentrations of N_2-GO-HA NC on cell viability (%) (n=3 and Mean± S.E.)

Post exposure period (hrs)	Cell viability % in different doses (µg/ml)				
	100	50	25	10	0
24	71.97±0.69^{a}	75.57±0.37^{b}	91.03±0.26^{e}	87.88±0.45^{d}	84.76±0.37^{c}
48	71.66±0.70^{a}	74.28±0.06^{b}	89.45±0.23^{e}	87.29±0.15^{d}	83.71±0.72^{c}
72	71.20±0.41^{a}	72.69±0.23^{b}	87.52±0.30^{d}	86.35±0.15^{d}	80.89±0.31^{c}

Mean values bearing superscript in row differed significantly from each other (P<0.01)

Table-2 Effect of different doses of N_2-GO-HA NC on PDT and MTT Assay of Caprine WJ-MSCs and Hemolysis (n=3 and Mean± S.E.).

Sr. No.	Parameters	Doses of N_2-GO-HA (µg/ml)				
		100	50	25	10	0
1	PDT (hrs)	51.04±1.71^{b*}	46.73±1.30^{b*}	39.97±1.32^{a*}	40.67±0.84^{a*}	41.70±1.87^{a*}
2	MTT assay (absorbance)	0.0429 ±0.0001^{a}	0.0512 ±0.009^{b}	0.0679 ±0.009^{c}	0.0678 ±0.0005^{c}	0.0677 ±0.0006^{c}
3	Hemolysis (%)	4.57±1.06^{b}	2.98±0.40^{ab}	2.23±0.27^{a}	2.18±0.25^{a}	1.31±0.14^{a}

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

Table-3 Effect of different doses of N_2-GO-HA on blood cells (n=3 and Mean± S.E.).

Blood cells	Doses of N_2-GO-HA NC (µg/ml)				
	100	50	25	10	0
RBCs (10^6/µl)	4.61±0.40	4.85±0.45	5.18±0.35	5.10±0.35	5.48±0.30
WBCs (10^3/µl)	10.20±0.23	10.17±0.20	10.47±0.15	10.80±0.20	11.40±0.30
Platelets 10^3/µl	161.00±17.56	165.67±24.03	167.33±23.18	163.33±4.37	196.67±22.39

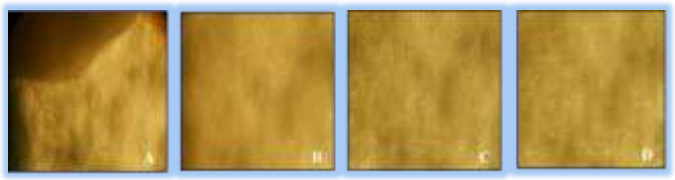


Fig-1 Caprine WJ-MSCs (A: Day 3, B: Day 7, C: Day 10 and D: Day 14)

Caprine WJ-MSCs were characterized by detecting Alkaline Phosphatase activity after 15 minute post staining [Fig-2].

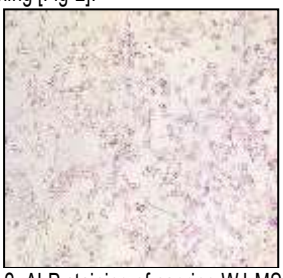


Fig-2. ALP staining of caprine WJ-MSCs

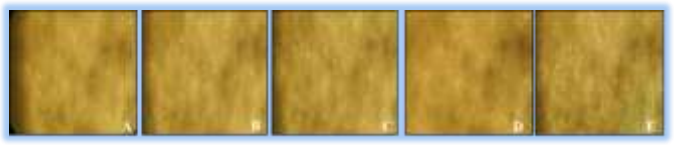
2. *In vitro* cytotoxicity assays

i. Cell morphology assay

Significant morphological alterations and detached round cells were observed in caprine WJ-MSCs treated with 100 and 50 µg/ml doses while no significant morphological changes were seen in doses 25 and 10 µg/ml and cells were normal like control on 24 and 48 hrs exposure. However, on 72 hrs exposure25 and 10 µg/ml dose treated caprine WJ-MSCs showed slightly better growth as compared to control group but in doses 100 and 50 µg/ml more number cells were floating and detached [Fig-3].

24 hrs incubation

48 hrs incubation



72 hrs incubation

Fig-3. Morphology of cWJ-MSCs exposed to N_2-GO-HA NC for 24, 48 and 72 hrs [A-100µg/ml, B- 50µg/ml, C-25µg/ml, D- 10µg/ml, E-0µg/ml (control)]

ii. Cell viability

Caprine WJ-MSCs viability increased significantly (P<0.01) in doses 25 and 10 µg/ml while significant (P<0.01) decrease was observed in doses 100 and 50 µg/ml as compared to control (0 µg/ml) after 24, 48 and 72 hrs exposure. However, highest cell viability was recorded in dose 25 µg/ml and lowest in dose 100 µg/ml throughout exposure duration which has no significant effect on cell viability [Table-1].

iii. Cell growth kinetic

In control group, caprine WJ-MSCs were followed normal growth pattern consisting with short lag phase followed by log phase of rapid growth and stationary phase with declined growth rate. N_2-GO-HA NC in doses 25 and 10 µg/ml significantly (P<0.05) increased growth rate while 100 and 50 µg/ml doses significantly (P<0.01) reduced growth rate and changed shape of growth curve as compared to control group [Fig-4].

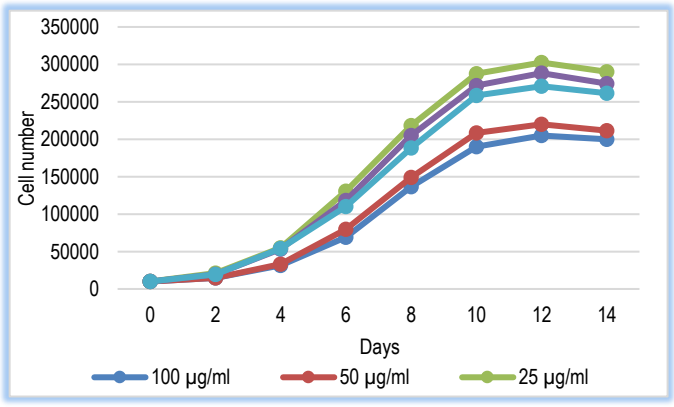


Fig-4 Growth curves of caprine WJ-MSCs exposed to different doses of N_2-GO-HA NC

iv. Population doubling time (PDT)

Caprine WJ-MSCs were doubled in 41.70±1.87 hrs (control *i.e.*, 0 µg/ml) and doubling time was decreased non-significantly in 25 and 10 µg/ml doses as compared to control. However, in doses 100 and 50 µg/ml significant ($P<0.05$) increase in PDT as 51.04±1.71and 46.73±1.30hrs respectively recorded and highest PDT recorded in dose 100 µg/ml [Table-2].

v. MTT assay

The MTT assay absorbance values on exposure to 25 and 10 µg/ml doses did not significantly vary however 100 and 50 µg/ml doses showed significant ($P<0.01$) decrease in mean absorbance values in comparison to control with lowest values in dose 100 µg/ml [Table-2].

vi. Hemolysis assay

No significant difference in % hemolysis was reported in doses 50, 25, 10 and 0µg/ml but highest % hemolysis was recorded in dose 100 µg/ml. Significantly ($P<0.01$) higher hemolysis was observed in doses 100 µg/ml [Table 2] after 4 hrs incubation as compared to control.

vii. Blood cell responses

Mean RBCs, WBCs and platelets count showed no significant changes in all doses as compared to control. Mean RBCs, WBCs and platelets counts in control were recorded as 5.48±0.30x106/µl, 11.40±0.30x103/µl and 196.67±22.39x103 /µl blood respectively. However, slight numerical decreased in RBCs, WBCs and platelets count was observed in doses 100,50, 25 and10 µg/ml as compared to and control [Table-3].

Discussion

The present study showed that MSCs isolated from caprine Wharton's jelly exhibits typical fibroblast like structure as reported earlier [40 and 27]. Their morphological features are similar to MSCs isolated from bone marrow [41], cord blood, amniotic sac, WJ and amniotic fluid [42] in caprine. Caprine WJ-MSCs were positive for ALP staining as per previously reported findings in caprine by [37 and 27]. *In vitro* cytotoxicity analysis of N_2-GO-HA NC in caprine WJ-MSCs demonstrated as 100 and 50 µg/ml doses affects morphology of cells considerably as cells were detached from surfaces while 25 and 10 µg/ml doses appear biocompatible as there is minimum influence on cell morphology as compared to control group on 24, 48 and 72 hrs exposure. These results coincide with the previous reports in human Adipose Derived-MSCs cultured on 0.5 wt.% GO in GO-GHA (Graphene oxide gelatin hydroxyapatite scaffolds) [43], caprine WJ-MSCs exposed with GO-Fe_2O_3 NC [28] and GQDs [27], MC3T3-E1 cells exposed to rGO-nanosheets (rGO NSs) and rGO-HA NC [44] where lower doses not induced cytotoxicity. Human osteoblast cells showed polygonal morphology after 24 hrs exposure to 1.5 wt. % rGO in HA-rGO NC [35]. Multiwalled carbon nanotubes (MWCNTs) (80 µg/ml) [45], carbon nanotubes (CNTs), graphene and carbon black also induced cytotoxicity in dose dependent manner (120, 60, 30 and 15 µg/ml) in murine macrophage (RAW-264.7) cells [46]. Nanocrystalline glass like carbon powder induced cytotoxicity (50 µg/ml) in SN4741 cells on 24 hrs, 3 and 7 days [33]. N_2-GO-HA NC in 25 and 10 µg/ml doses significantly ($P<0.01$) increased caprine WJ-MSCs viability but in 100 and 50 µg/ml doses significantly ($P<0.01$) reduced viable cell number as compared to control group on 24, 48 and 72 hrs exposure. N_2-GO-HA NC in 25 and 10 µg/ml doses acts as stimulant as there is increased proliferation of caprine WJ-MSCs and exposure period did not affect significantly caprine WJ-MSCs in all doses including control group. Similar findings reported in RAW-264.7 cells as proliferation rate and viability was decreased on exposure to MWCNTs at 80 µg/ml dose [45], CNTs and graphene are also cytotoxic in RAW-264.7 cells in dose dependent manner [46], GO-HA and graphene-HA (G-HA) hybrid materials films increased viability of MC3T3-E1 cells [29], increased proliferation rate of human osteoblast cells cultured with HA-rGO composite powder and highest in 1.0 wt. % rGO in HA-rGO from 3 to 5 days [34]. GO promoted L929 and MG63 cell proliferation rate but dose of GO in Titanium (Ti)-HA composites determined *In vitro* cytotoxicity [47]. rGO-HA NC also did not alterMC3T3-E1 cell viability at dose 31.3 µg/ml [44]. Growth pattern and PDT of caprine WJ-MSCs are similar with the earlier findings in caprines [27, 28 and 42] and bovine [48]. N_2-GO-HA NC in 100 and 50 µg/ml doses significantly ($P<0.05$) affected on growth rate and PDT while 25 and 10 µg/ml doses increased growth rate and decreased PDT as compared to control. This clearly indicates that as dose of N_2-GO-HA NC significantly affect the growth rate and this is in accordance with the earlier studies in caprine WJ-MSCs exposed to GQD [27] and GO-Fe_2O_3 NC [28], 3D graphene hydrogel in MG63 cells [49] and human osteoblast cells cultured on sintered graphite [50]. Lower absorbance values of MTT assay as compared to control indicates that decrease in cell proliferation and cytotoxic effect of N_2-GO-HA NC in caprine WJ-MSCs. 25 and 10 µg/ml doses of N_2-GO-HA NC are not cytotoxic but high doses that is 100 and 50 µg/ml are significantly ($P<0.01$) cytotoxic which reduces cellular metabolism in caprine WJ-MSCs. The similar results were reported previously in caprine WJ-MSCs incubated with GQD [27], and RAW 264.7 cells under exposure of graphene and CNTs [46], GO hybrid nanoconstruct in HeLa and PC-3 cells [51], fabricated GO with silica in HeLa cells [52], human osteoblast cells exposed with HA-rGO NC [35] and HA-GN composite powder deposited on Ti substrates [36]. Increased proliferation of human dental follicle stem cells (hDFSCs) cultured with nitrogen doped graphene nanomaterial [53]. Caprine RBCs hemolyzed significantly ($P<0.01$) in 100 µg/ml doses whereas only slight hemolysis was recorded in 50, 25,10 µg/ml as compared to control group under exposure of N_2-GO-HA NC. RBCs, WBCs and platelets count only numerically decreased in all doses of N_2-GO-HA NC as compared to control group and similar results were reported in human erythrocytes incubated with Ag nanoparticles [54 and 55], SiO_2 nanoparticles [56] in higher doses. However, TiO_2 nanoparticles significantly ($P<0.05$) reduced all blood cells count in human peripheral blood and RBCs non significantly hemolyzed at certain doses of TiO_2 nanoparticles but osmatic fragility of RBCs did not alter even at 500 µg/ml dose [57] and acid oxidized SWCNTs did not damaged RBCs at 20 nmol/L [58].

Conclusion

Present study concludes that caprine WJ-MSCs exposed to lower doses (25 and 10 µg/ml) of N_2-GO-HA NC stimulate their growth and these doses are biocompatible however higher doses (100 and 50 µg/ml) have significant cytotoxic effects. Doping of nanomaterials with nitrogen and hydroxyapatite led to reduction in toxicity and increased biocompatibility of graphene oxide.

Application of research

The report generates the baseline data of *In vitro* cytotoxicity of N_2-GO-HA NC in caprine WJ-MSCs which can be useful in stem cell research and in the field of tissue engineering in future.

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References

[1] Nikalje A. P. (2015) *Medical Chemistry*, 5 (2), 81-89.

[2] Agrawal S. and Rathore P. (2014) International Journal of Current Microbial Applied Science, 3(3), 43-55.

[3] Seyedjafari E., Soleimani M., Ghaemi N. and Sarbolouki M. N. (2010) Journal of Material Science:Material *Medicine* 22(1),165-174.

[4] Bahadar H., Faheem M., Kamal N. and Mohammad A. (2016) Iranian Biomed Journal,20(1),1-11.

[5] Estelrich J., Escribano E., Queralt J. and Busquets M. A. (2015) *International Journal of Molecular Science*,16, 8070-8101.

[6] Green J. J., Zhou B. Y., Mitalipova M. M., Beard C., Langer R., Jaenisch R. and Anderson D. G. (2008) *Nano Letter*, 8, 3126-3130.

[7] Lobatto M. E., Calcagno C., Metselaar J. M., Storm G., Stroes E.S., Fayad Z. A. and Mulder W. J. (2012) *Methods Enzymol*,508, 211-228.

[8] Koh I. and Josephson L. (2009) *Sensors*, 9, 8130-8145.

[9] Patil U.S., Qu H. Caruntu D. O., Connor C. J., Sharma A., Cai Y. and Tarr M. A. (2013) *Bioconjugate Chemistry*, 24,1562-1569.

[10] Sawdon A., Weydemeyer E. and Peng C. A. (2014) *Journal of Biomedical Nanotechnology*, 10, 1894-1917.

[11] Jayakumar R., Ramachandran R., Divyarani V. V., Chennazhi K. P., Tamura H. and Nair S. V. (2011*) International Journal of Biological Macromolecular*,48, 336-344.

[12] Krishna L., Dhamodaran K., Jayadev C., Chatterjee K. Shetty, R., Khora S. and Das D. (2016) *Stem Cell Research & Therapy*, 7,188.

[13] Chao K. C., Chao K. F., Fu Y. S. and Liu S. H. (2008)*PLoS ONE*,3(1), e1451.

[14] Noort W. A, Feye D., Akker F., Stecher D., Chamuleau S. A., Sluijter J. P. and Doevendans P. A. (2010) *Panminerva Medica*,52,27-40.

[15] Weiss M. L., Medicetty S., Bledsoe A. R., Rachakatla R. S., Choi M., Merchav S., Luo Y., Rao M. S., Velagaleti G. and Troyer D. (2006) *Stem Cells*,24(3),781-792.

[16] Yan Y., Xu W., Qian H., Si Y., Zhu W., Cao H., Zhou H. and Mao F. (2009) *Liver International*,29(3),356-365.

[17] Batsali A. K., Kastrinaki M. C., Papadaki H. A. and Pontikoglou C. (2013) *Current Stem Cell Research & Therapy*, 8(2),144-155.

[18] Yadegar M., Hekmatimoghaddam S. H., Saridar S. N. and Ali J. (2015) *Iranian Journal of Reproduction Medicine*, 13(3),141-148.

[19] Nguyen K. T. (2013) *Journal of Nonmedical Nanotechnology*,4, 128.

[20] Kaur S. and Singhal, B. (2012)*Journal of Bioscience and Bioenergy*,113(1):1-4.

[21] Parak W. J., Gerion D., Pellegrino T., Zanchet D., Micheel C., Williams C. S., Boudreau R., LeGros M. A., Larabell C. A and Alivisatos A. P. (2003) *Nanotechnology*,14(7),15-27.

[22] Elkhenany H., Lisa, A., Andersen L., ShawnB., Mark C., Nancy N., Enkeleda D., Oshin D. Alexandru S., Biris D. A. and Dhar M. (2015) *Journal of Applied Toxicology*, 35, 367-374.

[23] Arghya N. B. (2016) *Global Journal of Nanomedicine*, 1(1),555552.

[24] Nishida E., Miyaji H., Takita H., Kanayama I., Tsuji M., Akasaka T., Sugaya T., Sakagami R. and Kawanami M. (2016) *International Journal of Nanomedicine*, 11, 2265-2277.

[25] Yang Y., Asiri A.M., Tang Z., Du D. and Lin Y. (2013) *Materials Today*, 16(10), 365-373.

[26] Wang H., Maiyalagan T. and Wang X. (2012) *ACS Catalysis*, 2(5),781-794.

[27] Dar R. M., Gade N. E., Mishra O. P., Khan J. R., Vinod K. and Patiyal A. (2015) *Journal of Cell& Tissue Research*,15(1),4703-4710.

[28] Gade N. E., Dar R. M. Mishra O. P., Khan J. R., Vinod K. and Patyal A. (2015) *Journal of Animal Research*, 5(3),415-421.

[29] Kim S., Sook H. K., Lim S.Y., Kim J. H. and Park C. B. (2011) *Advanced Materials*,23(17),2009-2017.

[30] Wang J., Sun P., Bao Y., Liu J. and An L. (2011*) Toxicology In vitro*, 25(1),242-250.

[31] Nayak T. R., Henrik A., Venkata S. M., Clement K., Sukang B., Xiangfan X., Pui L. R., Jong H. A., Byung H. H., Giorgia P. and Barbaros O. (2011)*ACS Nano*,5(6),4670-4678.

[32] Wadhwa S., Rea C., Hare P. O., Mathur A., Roy S. S., Dunlop P. S., Byrne J. A., Burke G., Meenan B. and McLaughlin J. A. (2011) *Journal of Hazardous Materials*, 191(1-3),56-61.

[33] Rodriguez L. N., Romero P., Estivill T. G., Guzma V. R. and Aguirre J. A. (2017) *PLoSONE*,12(3), e0173978.

[34] Liu Y., Huang J. and Li H. (2013) *Journal of Materials Chemistry B*,1(13),1826-1834.

[35] Baradaran S., Moghaddam E., Basirun W. J., Mehrali M., Sookhakian M., Hamdi M., Nakhaei-Moghaddam M. R. and Alias Y. (2014) *Carbon*, 69, 32-45.

[36] Liu Y., Dang Z., Wang Y., Huang J. and Li H. (2014) *Carbon*, 67,250-259.

[37] Babaei H., Moshrefi M., Golchin M. and Mematollahi-Mahani S. N. (2008) *Iranian Journal of Veterinary Surgery*, 3(2),57-65.

[38] Bregoli L., Chiarini F., Gambarelli A., Sighinolfi G., Gatti A. M., Santi P., Martelli A. M. and Cocco L. (2009) *Toxicology*, 262(2),121-129.

[39] Vinjamuri S., Shanker D., Rao S. R. and Nagarajan S. (2015) *World Journal of Pharmacy and Pharmaceutical Sciences*, 4(7):1263-1268.

[40] Moshrefi M., Babaei H., and Nematollahi-Mahani S. N. (2010) *Animal Reproduction*, 7(4),367-372.

[41] Remya V. Naveen K., Ninu A. R., Radhika S., Kutty M. V. H. and Maity S. K. (2014) *Indian Journal of Science Research &Technology*,2(3),60-62.

[42] Somal A., Bhat I. A., Indu,B., Pandey S., Panda B. S. K., Thakur N., Sarkar M., Chandra V., Saikumar G. and Sharma G. T. (2016) *PLoSONE*,11(6),1-17. e0156821.

[43] Nair, M., Nancy, D., Krishnan, A. G., Anjusree, G. S., Vadukumpully, S. and Nair, S. V. (2015) *Nanotechnology*, .26(16),161001.

[44] Lee J. H., Shin Y. C., Lee S. M., Jin O. S., Kang S. H., Hong,S. W., Jong C. M., Huh J. B. and Hon D. W. (2015) *Scientific Reports*,5,18833.

[45] Szczypta A. F., Menaszek E., Syeda T. M., Mishra A., Alavijeh M., Adu J. and and Blazewicz S. (2012) *Journal of Nanoparticle Research*, 14,1181.

[46] Figarol A., Jeremie P., Delphine B., Valerie F., Celine A., Jean M. T., Jean P. L., Michele C., Didier B. A. and Philippe G. (2015) *Journal of Toxicology In vitro*,9(14).

[47] Li M., Liu Q., Jia Z., Xu X., Cheng Y., Zheng Y., Xi T. and Wei S. (2014) *Carbon*, 67,185-197.

[48] Gade N. E., Pratheesh M. D., Nath A., Dubey P. K., Amarpal Sharma B., Saikumar G. and Sharma G. T. (2012)*Reproduction in Domestic Animals*, 48(3),358-367.

[49] Lim H. N., Huang N. M., Lim S. S., Harrison I. and Chia C. H. (2011) *International Journal of Nanomedicine*, 6,1817-1823.

[50] Czarnecki J. S., Lafdi K. and Tsonis P. A. (2008) *Biology Faculty Publications. Paper* 12.

[51] Kim H., Namgung R., Singha K., Oh I. K. and Kim W. J. (2011) *Bioconjugate Chemistry* 22(12),2558-2567.

[52] Sreejith S., Ma X. and Zhao Y. (2012)*Journal of American Chemical Society*,134 (42),17346-17349.

[53] Olteanu D., Filip V., Socaci V., Biris A. R., Filip X., Coros M., Rosu M. C., Pogacean F., Alb C., Baldea I., Bolfa P. and Pruneanu S. (2015) *Colloids Surface B:Biointerfaces*, 136,791-798.

[54] Choi J., Reipa V., Hitchins V. M., Goering P.L., and Malinauskas R. A. (2011) *Toxicological Sciences*, 123(1),133-143.

[55] Laloy J., Minet V., AlpanL., Mullier F., Beken S., Toussaint O., Lucas S. and Dogne J. M. (2014)*Nanobiomedicine*, 1,4.

[56] Zhao Y., Sun X., Zhang G., Trewyn B. G., Slowing S. I. and Lin S. Y. (2011) *ACS Nano*,5(2),1366-1375.

[57] Ghosh M., Chakraborty A. and Mukherjee A. (2013) *Journal of Applied Toxicology*, 33(10),1097-1110.

[58] Donkor A. D., Su Z., Mandal H. S., Jin X. and Tang X. (2009) *Nano Research*, 2,517-525.