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In vitro cytocompatibility assessment of nitrogen doped graphene oxide gold (N_2-GO-Au) nanohybrid in caprine wharton's jelly derived mesenchymal stem cells (WJ-MSCs) and erythrocytes

SA Dhenge, NE Gade, OP Mishra, Abinash Kumar and VN Khandait

Abstract
Nanomaterials are applied in stem cell culture and *in vivo* transplantation as well as tracking and in this study nitrogen doped graphene oxide gold (N_2-GO-Au) nanohybrid dose dependent (100, 50, 25, 10 and 0 µg/ml) *in vitro* cytocompatibility was analysed in caprine Wharton's jelly derived mesenchymal stem cells **(**WJ-MSCs) and erythrocytes. Caprine WJ-MSCs were grew in colonies and exhibited fibroblastic morphology. N_2-GO-Au nanohybrid was greatly disturbed cell morphology, significantly ($P< 0.01$) decreased viability and proliferation rate and significantly ($P< 0.05$) increased population doubling time (PDT) at 100 and 50 µg/ml doses, but unchanged cell morphology and significantly ($P< 0.01$) increased viability and proliferation rate was observed at 25 and 10 µg/ml doses as compared to control. However, N_2-GO-Au nanohybrid did not alter cell PDT and growth characteristics at 25 and 10 µg/ml doses and significantly ($P< 0.05$) declined growth was observed at 100 and 50 µg/ml doses as compared control and N_2-GO-Au nanohybrid did not hemolysed caprine erythrocytes. In conclusion, N_2-GO-Au nanohybrid was cytocompatible at 25 and 10 µg/ml doses and cytotoxic at 100 and 50 µg/ml doses in caprine WJ-MSCs alongwith no hemolytic effect on erythrocytes.

Keywords: Nanotechnology, graphene, cytotoxicity, stem cells, caprine

Introduction
Nanotechnology is applied in biosciences including stem cell research and stem cells are used to treat regenerative diseases [1] as stem cells are self-renewable and differentiate into organs cells. Stem cells are resides nearly in all adult tissue and Wharton's jelly (WJ) derived mesenchymal stem cells (MSCs) is a reliable stem cell source and it can be isolated without ethical consideration. However, there are certain challenges in stem cell culture, maintenance, differentiation and *in vivo* transplantation as well as tracking. Therefore, to overcome this great challenge, nanomaterials are applied in stem cell research [2] and these research areas have been attracted many researchers to analyze nanomaterials potency and biocompatibility in stem cells. Carbon based nanomaterials (CBNs) such as graphene, graphene oxide (GO), reduced GO etc. were stimulated stem cell growth and differentiation [3,4] as well as exhibited biocompatibility [5]. However, heteroatoms doped GO derivatives exploit GO properties [6] and these hybrid materials were revealed dose dependent biocompatibility in stem cells [7, 8]. The dose and time dependent GO [9] and gold (Au) nanoparticles [10] *in vitro* cytotoxicity was observed in stem cells. However, N_2 doped GO with Au nanoparticles (N_2-GO-Au) nanohybrid cytocompatibility till was not investigated in animal stem cells and blood cells. This study was hypothesized as N_2doped GO enhances the GO properties and reduces Au nanoparticles cytotoxicity in caprine WJ-MSCs and erythrocytes. Hence, the present study was planned to assess dose dependentN_2-GO-Au nanohybrid cytocompatibility in caprine WJ-MSCs and erythrocytes.

Materials and Methods
Isolation of caprine WJ-MSCs and erythrocytes: Present study protocol was approved by Institution Animal Ethics Committee and this study was carried out at Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal

Husbandry, Anjora, Dist. Durg (C.G.).Gravid caprine uteri (~45 days) were collected from local abattoir and caprine WJ-MSCs were isolated [11]. In brief, umbilical cord was washed in sterile phosphate buffer saline and cut longitudinally to remove blood vessels with care and remaining tissue as Wharton's jelly (WJ) sliced into small fragments (1-2 mm^2) and first washed in Dulbecco's phosphate buffer saline(DPBS) and then Dulbecco's modified eagle's medium (DMEM) supplemented with 15%fetal bovine serum. WJ fragments were plated in tissue culture flask and incubated in CO_2 incubator at 37^0 C and 5% CO_2environment and jelly fragments were removed on day 5^{th} and media was changed initially on day 5^{th} and thereafter every 4^{th} day. Caprine WJ-MSCs were observed periodically, passaged and third passaged cells were used to study cell cytotoxicity assays. In EDTA coated tube 5.0 ml blood was collected through jugular vein puncture from adult female caprine and erythrocytes were isolated [12]. Cell cytotoxicity assays were carried out to assessN_2-GO-Au nanohybrid cytocompatibility at doses 100, 50, 25, 10 and 0 µg/ml.

Cell morphology assay: Caprine WJ-MSCs were cultured in 24 well cell culture plate and confluent cell monolayer was treated with N_2-GO-Au nanohybrid as per mentioned doses and morphological changes were observed every after24hrsintervals during 3 consecutive days.

Cell viability: Caprine WJ-MSCs were incubated with different doses of N_2-GO-Au nanohybrid in 24 well cell culture plates and cell viability was determined by trypan blue dye exclusion test [13] after72 hrs.

Cell growth kinetic (GK): Cell growth kinetic assay was carried out to study caprine WJ-MSCs growth characteristics withN_2-GO-Au nanohybrid treatment [8].

Cell Population doubling time (PDT): Caprine WJ-MSCs were incubated with respective doses of N_2-GO-Au nanohybrid in 24 well cell culture plates and cell PDT was determined[8] as follows, PDT= Culture time (CT)/Cell doubling (CD), where, CD=log (NH/NI)/ log2, NH is harvested cell number and NI is initial cell number

Tetrazolium dye 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) MTT assay: MTT assay was carried out to assessN_2-GO-Au nanohybrid effect on caprine WJ-MSCs proliferation rate. Freshly harvested caprine WJ-MSCs ($1x10^6$ cells / ml) were incubated with N_2-GO-Au nanohybrid at different doses in wells of 96 well cell culture plate and after 24 hrs incubation cell monolayer was treated with MTT reagent and incubated for 4 hrs in CO_2 incubator. Cells were observed periodically to develop dark colour MTT formazan crystals precipitate and it was dissolved with 100 µl solubilization solution. MTT formazan crystals were dissolved completely by gentle stirring and absorbance was read at 620 nm on ELISA plate reader and absorbance value is directly proportional to viable cell number.

Hemolysis assay: Hemolysis assay was carried out to study N_2-GO-Au nanohybrid cytocompatibility in caprine erythrocytes. Isolated caprine erythrocytes in DPBS were incubated with N_2-GO-Au nanohybrid at different doses in microcentrifuge tubes for 4 hrs at 37^0C in incubator and negative as well as positive controls were also maintained. All samples were centrifuged and 100 µl haemoglobin in supernatant was transferred in flat bottom 96 wells tissue culture plate with care and absorbance was read at 492 nm in ELISA plate reader and hemolysis% was determined with following formula,

$$\text{Hemolysis \%} = \frac{\text{Test sample absorbance} - \text{Negative control absorbance}}{\text{Positive control absorbance}} \times 100$$

Statistical analysis: ata recorded in present study was expressed as mean ± standard error (S. E.) values and One-way ANOVA was applied using IBM SPSS Statistics 25 software and values of $P< 0.01$ and $P < 0.0$ were considered statistically significant.

Results

Caprine WJ-MSCs were grew in periphery of jelly fragments on day 3 and were exhibited spindle shape small cells with large nucleus and elongated morphology and mostly fibroblastoid morphology and cells were reached confluent stage on day 14 (Fig. 1).

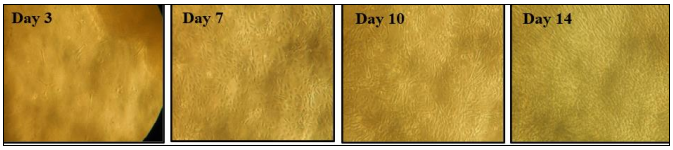

Fig 1: Caprine WJ-MSCs during different intervals (3-14 days)

Cell morphology: Cell morphology was significantly disturbed at doses 100 and 50 µl/ml N2-GO-Au nanohybrid but, significant cell growth with normal fibroblast morphology was observed at doses 25 and 10 µl/ml N_2-GO-Au nanohybrid as compared with control (Fig. 2).

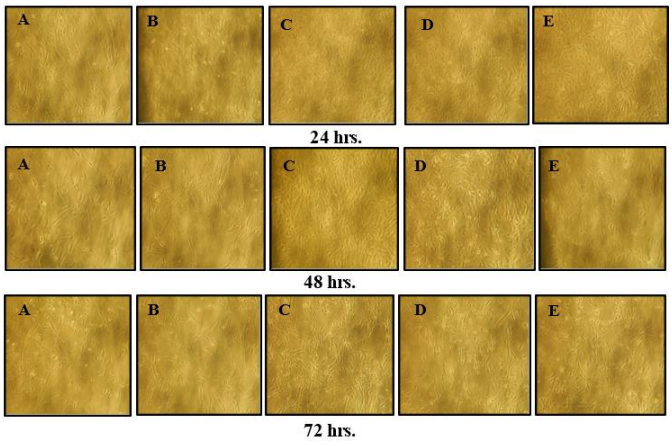

Fig 2: Caprine WJ-MSCs morphology (A-100 µg/ml, B- 50 µg/ml, C- 25 µg/ml, D-10µg/ml and E-0 µg/ml)

Cell viability: N_2-GO-Au nanohybrid significantly ($P< 0.01$) decreased cell viability at 100 and 50 µg/ml doses and significantly ($P< 0.01$) increased cell viability was observed at 25 and 10 µg/ml as compared with control (Table 1).

Cell growth kinetic: Cells in control group were followed normal growth pattern consisting initial slow growth of lag phase (0-2 days), log phase of exponential growth (4-8 days) and stationary phase (10-12 days) with declined growth (12-14 days) during 0 to 14 days (Fig 3). N_2-GO-Au nanohybrid at doses 100 and 50 µg/ml significantly ($P< 0.05$) altered

shape of growth curve (declined) as compared to 25, 10 and 0 µg/ml doses however, N_2-GO-Au nanohybrid at 25 µg/ml dose significantly ($P< 0.05$) changed shape of growth curve (inclined) (Fig 3).

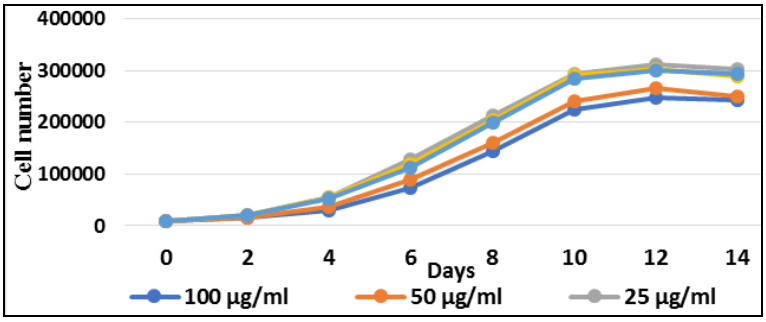

Fig 3: Effect of N_2-GO-Au nanohybrid on caprine WJ-MSCs growth curve

Cell PDT: Caprine WJ-MSCs in control group were doubled in 42.01±2.36 hrs and N_2-GO-Au nanohybrid at 10 and 25 µg/ml doses did not alter cell PDT but, at 100 and 50 µg/ml doses cell PDT was significantly ($P< 0.05$) increased as compared to control (Table 1).

Table 1: Effect of N_2-GO-Au nanohybrid on caprine WJ-MSCs viability, PDT, MTT assay absorbance value and hemolysis% (Mean ± S.E.)

Sr. No.	Doses (µg/ml)	Cell viability%	PDT (hrs)	Absorbance value	Hemolysis%
1.	100	72.63±0.16a	51.45±3.01b*	0.0430±0.0002a	3.92±0.53
2.	50	74.32±0.19b	49.75±2.62b*	0.0509±0.0004b	2.69±0.80
3.	25	84.56±0.18e	38.70±1.89a*	0.0673±0.0007d	2.70±0.57
4.	10	82.32±0.17d	39.54±1.41a*	0.0676±0.0015d	1.99±0.43
5.	0	80.12±0.06c	42.01±2.36a*	0.0621±0.0019c	1.33±0.03

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

MTT assay: MTT assay absorbance value of caprine WJ-MSCs treated with N_2-GO-Au nanohybrid at 100 and 50 µg/ml doses significantly ($P< 0.01$) decreased while, at 10 and 25 µg/ml doses it was significantly ($P< 0.01$) increased as compared to control (Table 1).

Hemolysis assay: Caprine erythrocytes were not significantly hemolysed by N_2-GO-Au nanohybrid at all doses as compared with control, however, dose dependent hemolysis was observed (Table 1).

Discussion

Caprine WJ-MSCs isolated and cultured successfully in present study and cells were grew in colonies and exhibited mostly fibroblast like morphology [8,14] and similar morphology was also reported in BM-MSCs in caprine [4] and buffalo [15]. In N_2-GO-Au nanohybrid material, GO a principal composite which boosted morphology and biocompatible in MC3T3-E1 cells [16], human neural stem cells (hNSCs)[17], human adipose tissue derived stem cells (AD-MSCs)[9]and murine MSCs [5].In present study, N_2-GO-Au nanohybrid at 100 and 50 µg/ml doses considerably disturbed cell morphology which demonstrated their cytotoxicity in caprine WJ-MSCs while, at 25 and 10 µg/ml doses stimulated growth which showed their cytocompatibility. Dose dependent alterations in caprine WJ-MSCs morphology by N_2-GO-Au nanohybrid in these study is similar as like previous reports in caprine WJ-MSCs[4,7,8], human osteoblast cultured on rGO film (0.5, 1.0 and 1.5 wt.%)[18], MC3T3-E1 cells seeded in 3D GO (0.1 to 1.0 µg/ml) collagen scaffolds [19]. Also, human MSCs morphology did not alter that cultured on graphene coated SiO_2, polymethyl siloxane, polyethylene terephthalate substrates [20]. However, multiwalled carbon nanotubes (MWCNTs) (80 µg/ml) [21], carbon powder (50 µg/ml) in SN4741 cells [22], carbon nanotubes (CNTs), graphene and carbon black (120, 60, 30 and 15 µg/ml) in murine macrophage (RAW-264.7) cells induced cytotoxicity in dose dependent manner[23].N_2-GO-Au nanohybrid was stimulated caprine WJ-MSCs viability and proliferation rate at 25 and 10 µg/ml doses, however, decreased cell proliferation rate and viability was observed at 100 and 50 µg/ml doses. These results are coinciding with earlier reports in murine MSCs cultured on GO (1.0 mg/ml) coated glass substrates [5], graphene quantum dots (GQDs) [7] and GO-Iron oxide nanocomposites (GO-Fe_2O_3NC) [8] in caprine WJ-MSCs. However, pristine graphene did not alter human MSCs viability [20] and pure Au nanoparticles did not induce considerable cytotoxicity in chick embryo fibroblast cells and HeLa cells up to doses 50 and 40 µg/ml respectively, over 24 hrs exposure [24] which are not accordance with present study findings, as ≥50 µg/ml doses of N_2-GO-Au nanohybrid are cytotoxic in caprine WJ-MSCs. Doping of GO with N_2 and their Au nanoparticles nanohybrid might be improved GO and Au nanoparticles properties in caprine WJ-MSCs as pure Au particles tolerates human AD-MSCs viability only at 10 µg/ml [10] and 0.004 µg/ml or 0.065 µg/ml [25] doses. In addition, N_2nanoparticles might be reduced Au nanoparticles cytotoxicity and exhibited biocompatibility up to 25 µg/ml doses which was previously justified as functionalized Au nanoparticles with organic nanoparticles was improved human MSCs viability [26]. Caprine WJ-MSCs growth characteristics and PDT in control group are accordance with earlier findings in caprine [7, 8, 14] and bovines [15] but, N_2-GO-Au nanohybrid at 100 and 50 µg/ml doses was significantly ($P< 0.05$) affected on cell growth pattern and PDT as compared to control and significant ($P< 0.05$) reduced cell PDT was observed at 25 and 10 as compared to 100 and 50 µg/ml doses. In this study the N_2-GO-Au nanohybrid at high doses (100 and 50 µg/ml) was cytotoxic but, low doses (25 and 10 µg/ml) showed biocompatibility with improved cell growth characteristics as like in caprine WJ-MSCs were treated with GQDs [7] and GO-Fe2O3 NC [8], GO-HA NC in

human osteoblasts [18] and Au nanoparticles was increased human AD-MSCs proliferation and differentiation rate [10]. MTT assay absorbance value is directly proportional to viable cell number indicating enhance quantitative cell viability and proliferation rate. In present study, N_2-GO-Au nanohybrid was significantly ($P< 0.01$) decreased absorbance value at 100 and 50 µg/ml doses which showed cytotoxic effect in caprine WJ-MSCs, but at 25 and 10 µg/ml doses significantly ($P< 0.01$) increased absorbance value which stimulated cell proliferation rate as compared to control. These findings are like BM-MSCs cultured on GO coated tissue culture plate [4], caprine WJ-MSCs treated by GQDs [7], GO (1.0 mg/ml) coated glass substrates were exhibited biocompatibility in murine MSCs [5], rGO and graphene were significantly increased rat NSCs proliferation rate and expressed neuronal (Tuj1) and glial cell (GFAP) surface markers [2]. Functionalized Au nano tracers were significantly increased human MSCs proliferation and differentiation [26] however, graphene and carbon nanotubes induced dose dependent (120, 60, 30 and 15 µg/ml) cytotoxicity in RAW- 264.7 cells [23] like present study findings. In this study, N_2-GO-Au nanohybrid dose is considerable issue as 100 and 50 µg/ml doses are inhibited caprine WJ-MSCs proliferation rate and lower doses (25 and 10 µg/ml) are stimulated their proliferation rate. In present study, slight hemolysis was occurred by N_2-GO-Au nanohybrid in caprine erythrocytes at all doses as compared to control because, GO relatively non cytotoxic in peripheral blood mononuclear cells at 200 µg/ml dose [27] and Au nanoparticles did not alter blood parameters (HB, PCV, RBCs and WBCs) in mice [28] as like present study. In addition, TiO_2nanoparticles were not cytotoxic even at 500 µg/ml in human red blood cells [29] as well as <1% hemolysis was occurred by Ag nanoparticles at 100 µg/ml dose [30] which are in support to present study findings.

Conclusion

The present study was concluded as, N_2-GO-Au nanohybrid was cytocompatible at 25 and 10 µg/ml doses and cytotoxic at 100 and 50 µg/ml doses in caprine WJ-MSCs, however, there was no any impact on caprine erythrocytes. Therefore, N_2-GO-Au nanohybrid (25 and 10 µg/ml doses) can be used in stem cell research.

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