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In vitro CYTOTOXIC EFFECTS OF HYBRID GRAPHENE OXIDE NANOCOMPOSITES (hGONCs) ON CRYOPRESERVED CAPRINE WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS (WJ-MSCs)

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ABSTRACT

In present study, caprine Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) were isolated and characterized by osteogenic and adipogenic differentiation. Total 12 hybrid graphene oxide nanocomposites (hGO NCs) such as N₂ doped GO-HA NCs, P doped GO-HA NCs, S doped GO-HA NCs, N₂ doped GO-SiO₂ NCs, P doped GO-SiO₂ NCs, S doped GO-SiO₂ NCs, N₂ doped GO-TiO₂ NCs, P doped GO-TiO₂ NCs, S doped GO-TiO₂ NCs, N₂ doped GO-Au NCs, P doped GO-Au NCs and S doped GO-Au NCs were incorporated at doses 100, 50, 25, 10 and 0 µg/ml in vitrification solution. Caprine WJ-MSCs were cryopreserved by using open pulled straw (OPS) vitrification method and analysed hGO NCs effect on their post thaw viability and culture characteristics. Caprine WJ-MSCs were exhibited normal fibroblastoid morphology and differentiated in to osteogenic and adipogenic lineages. The significant (P<0.01) highest and lowest caprine WJ-MSCs post thaw viability (cumulatively) was observed in P-GO-TiO₂ NC and P-GO-HA NC groups, respectively along with all doses (cumulatively) significantly (P<0.01) decreased post thaw cell viability as compared with control. All hGO NCs were significantly (P<0.01) decreased caprine WJ-MSCs post thaw viability at doses 100 and 50 µg/ml as compared to 25, 10 and 0 µg/ml doses. Post thaw caprine WJ-MSCs were grew in normal pattern with similar fibroblast like morphology on days 14. In conclusion, all hGO NCs at doses 50 and 100 µl/ml are cytotoxic and P-GO-TiO₂ NC is least decrease caprine WJ-MSCs post thaw viability.

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1 Introduction

Nanotechnology is the study of materials and structures which are manipulate at nanoscale level and usually, a deliberately engineered material with at least one dimension 1-100 nm is called as nanomaterial (Hornyak et al., 2008). It is applied in different fields such as chemistry, energy, textile, cosmetics, space research, information technology, biomedicine and agricultural. Recently various types of nanomaterials are widely used in biomedicine for drug discovery (Estelrich et al., 2015) and delivery (Ambwani et al., 2018), bio-imaging (Lobatto et al., 2012), tissue engineering and regenerative medicine (Jayakumar et al., 2011), proteomics (Patil et al., 2015), genomics (Basu et al., 2013), *in vitro* disease diagnosis (Mamaeva et al., 2018) along with treatment (Kamaly et al., 2016) as well as in stem cell research (Deb et al., 2012). In stem cell research, nanotechnology is applied in stem cell culture (Goto et al., 2007), expansion, differentiation and transplantation (Guo et al., 2017), nano delivery of DNA, RNAi and proteins in stem cells differentiation (Park et al., 2011) as well as *in vivo* delivery (Nguyen, 2013) and imaging of stem cells (Michalet et al., 2005). Stem cells are specialized progenitor cells residing in nearly all organ tissue and have enormous potency to self-renew and repairs damage cells and differentiate *in vitro* into their lineage cells. Mesenchymal stem cells (MSCs) are adult stem cells and can be isolated from bone marrow and fetal adnexa tissue and differentiate into osteocytes, adipocytes and chondrocytes (Pittenger et al., 1999), pancreatic cells (Saman et al., 2014), hepatocytes (Borhani et al., 2015) and were used to treat numerous regenerative diseases (Sangeetha et al., 2017). Fetal adnexa derived MSCs can be isolated easily and Umbilical cord Wharton's jelly derived MSCs (WJ-MSCs) expand rapidly and differentiate into osteocytes, adipocytes and chondrocytes (Somal et al., 2016; Somal et al., 2017). Carbon-based nanomaterials (CBNs) are considered as excellent nanomaterials in tissue engineering (Oprych et al., 2016) and other biological applications (Yang et al., 2007) due to their relative biocompatibility. Graphene (GN) is an atom thick pure carbon monolayer arranged in two-dimensional honeycomb structure (Allen et al., 2010) and its derivatives as graphene oxide (GO) and reduced graphene oxide (rGO) were applied in biotechnology (Kim et al., 2011). However, dose and exposure time depended cytotoxicity of hybrid graphene nanomaterials i.e. graphene-based nanomaterials (GBNs) was reported in A549 lung epithelial cells (Wadhwa et al., 2011), 264.7 RAW macrophage (Szczypta et al., 2012; Figarol et al., 2015) and caprine WJ-MSCs (Dar et al., 2015; Gade et al., 2015). In spite this, certain GBNs were showed biocompatibility in human osteoblast (Baradaran et al., 2014), mouse osteoblastic MC3T3-E1 cells (Nishida et al., 2014), caprine BM-MSCs (Elkhenany et al., 2015) and murine MSCs (Kim et al., 2018) in dose dependent manner. In addition,

GO hybridization or doping with organic or inorganic nanomaterials or nanoparticles exploit their unique properties (Wang et al., 2012) and biocompatibility (Nishida et al., 2014) in stem cells. *In vitro* maintenance of stem cells is difficult task as it changes in its genotype and phenotype, lead to senescence and transformation as well as contaminated in incubator. Hence, stem cells cryopreservation technique can provide potential and regular stem cells source, save time and also culture materials. Stem cells were cryopreserved using vitrification by opened pulled straw (OPS) method (Bahadori et al., 2009), but post thaw survival rate is so variable, however, nanoparticles incorporation in cryoprotectant solution might improve efficiency of vitrification solution by changing its chemical properties (Li et al., 2016; Xu et al., 2016). However, best of our knowledge, *invitro* efficacy of hybrid graphene oxide (hGO) as GO doped with nitrogen (N₂), phosphorus (P) and sulphur (S) and their nanocomposites with hydroxyapatite (HA), Silica (Si), Titanium oxide (TiO₂) and gold (Au) were not studied instem cells. hGO NCs may be useful in stem cell research, regenerative medicine and also stem cells post thaw survivability rate can be increased by their incorporation in vitrification solution. Hence, *in vitro* efficacy of hGONCs in animal stem cell model is an important and therefore, present study was carried with the objective to study comparative *in vitro* effect of hGO NC soncaprine WJ-MSCs by studying post thaw cell viability.

2 Materials and Methods

Present study was conducted in Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Anjora, Durg (C.G.)-491 001 India and study protocol approved by Institution Animal Ethical Committee (No. VPB/PhD-1/2017).

2.1 Isolation of caprine WJ-MSCs

Gravid caprine uteri (45-60 days) were collected from nearby slaughter house and uteri were transported within 1 hr to Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Anjora, Durg, Chhattisgarh, India. Uteri were washed thoroughly by sterile saline and aseptically 2-3 cm long umbilical cord was cut and proceeded to isolate WJ-MSCs (Babaei et al., 2008). Caprine WJ-MSCs were cultured in Dulbecco's modified eagles' medium (DMEM) fortified with 15% Fetal Bovine Serum (FBS) and kept in CO₂ incubator at 37°C with 5% CO₂. Cells were observed periodically under inverted microscope and culture media was changed every after 4th days and cells were passaged on day 14 and third passaged caprine WJ-MSCs were characterized with Alzarin red and Oil red O staining (Baghaban et al., 2008) to test their osteogenic and adipogenic differentiation potential, respectively.

2.2 Hybrid graphene oxide nanocomposites (hGO NCs)

Total 12 hGO NCs such as N₂ doped GO-HA NCs, P doped GO-HA NCs, S doped GO-HA NCs, N₂ doped GO-SiO₂NCs, P doped GO-SiO₂NCs, S doped GO-SiO₂NCs, N₂ doped GO-TiO₂NCs, P doped GO-TiO₂NCs, S doped GO-TiO₂NCs, N₂ doped GO-Au NCs, P doped GO-Au NCs and S doped GO-Au NCs were generously supplied by Department of Physics, BHU, Banaras, Uttar Pradesh (India). hGO NCs were sterilized and total 5 treatment groups with different doses (100 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml and 0 µg/ml) were constituted to study effect of hGO NCs on caprine WJ-MSCs post thaw viability.

2.3 Cryopreservation of caprine WJ-MSCs

Third passaged caprine WJ-MSCs were cryopreserved with hGO NCs by using open pulled straw (OPS) vitrification method (Vajta et al., 1997; Vajta et al., 1998) with some modifications. Cells were harvested and cryopreserved by using equilibration [10 % Ethylene Glycol (EG) + 10 % Dimethyl Sulphoxide (DMSO)] and vitrification (20 % EG + 20 % DMSO + 0.5 M / L sucrose) solutions in DMEM supplied with 20% FBS. hGO NCs were added in vitrification solution as per designed doses to study cytotoxicity. Caprine WJ-MSCs transferred in media and suspended in equilibration solution for 5 minute and then vitrification solution mixed for 30 - 40 second. Cells droplets were loaded in sterilized French pulled straws by capillary effect and submerged immediately into liquid nitrogen and stored for 7 days. Cells were thawed and suspended in DMEM with 15% FBS and cell viability was assessed (Bregoli et al., 2009).

2.4 Statistical analysis

The data recorded in present study was analyzed with Full Factorial design and One way - ANOVA (IBM SPSS Statistics 25 software) and values are expressed as mean $\pm$ standard error (S. E.) and $P < 0.01$ and $P < 0.05$ are considered to be statistically significant.

3 Results

Caprine WJ-MSCs are plastic adherent and exhibited heterogenous morphology however, mostly fibroblastoid like cells were observed and reached confluent stage on day 14 (Figure 1).

Figure 1 Caprine WJ-MSCs in confluent stage on day 14

The third passage caprine WJ-MSCs were induced with respective differentiation media for 21 days to differentiate into osteocytes and adipocytes and stained positively with Alzarine red (figure 2A) and Oil red O staining (figure 2B), respectively.

Figure 2 Characterization of WJ-MSCs [A.] Alzarine red staining and [B.] Oil red O staining

Post thaw viability of caprine WJ-MSCs with hGO NCs in vitrification solution was analyzed after 7 days of cryopreservation. The cumulative significant ($P < 0.01$) highest and lowest caprine WJ-MSCs post thaw cell viability was observed in P-GO-TiO₂ NC and P-GO-HA NC added groups, respectively among all hGO NCs (Table 1). In addition, caprine WJ-MSCs post thaw viability significantly ($P < 0.01$) decreased in all doses of hGO NCs (cumulatively) as compared with control however, 100 and 50 µg/ml doses significantly ($P < 0.01$) decreased post thaw cell viability as compared with 25 and 10 µg/ml (Table 2).

Table 1 Cumulative effect of each hGO NCs on caprine WJ-MSCs post thaw viability

Sr. No.	hGO NCs	Post thaw viability %
1.	N ₂ -GO-HA NC	50.40 $\pm$ 1.74 ^{abc}
2.	P-GO-HA NC	45.93 $\pm$ 2.29 ^a
3.	S-GO-HA NC	49.93 $\pm$ 2.40 ^{abc}
4.	N ₂ -GO-SiO ₂ NC	50.53 $\pm$ 1.63 ^{abc}
5.	P-GO-SiO ₂ NC	51.33 $\pm$ 1.69 ^{bc}
6.	S-GO-SiO ₂ NC	49.53 $\pm$ 1.90 ^{abc}
7.	N ₂ -GO-TiO ₂ NC	46.60 $\pm$ 1.78 ^{ab}
8.	P-GO-TiO ₂ NC	51.53 $\pm$ 2.02 ^c
9.	S-GO-TiO ₂ NC	46.93 $\pm$ 2.05 ^{abc}
10.	N ₂ -GO-Au NC	46.53 $\pm$ 2.47 ^{ab}
11.	P-GO-Au NC	46.60 $\pm$ 2.50 ^{ab}
12.	S-GO-Au NC	47.87 $\pm$ 2.59 ^{abc}

Number of replicate = 15; Mean values bearing superscript in column differed significantly from each other ($P < 0.01$)

Table 2 Cumulative effect of each dose of hGO NCs on caprine WJ-MSCs post thaw viability

Sr. No.	Dose ($\mu\text{g/ml}$)	Post thaw viability %
1.	100	35.42 $\pm$ 0.71 ^a
2.	50	42.36 $\pm$ 0.81 ^b
3.	25	53.94 $\pm$ 0.61 ^c
4.	10	53.69 $\pm$ 0.53 ^c
5.	0	57.81 $\pm$ 0.46 ^d

Number of replicate =36; Mean values bearing superscript in column differed significantly from each other ($P<0.01$)

Figure 3 Caprine WJ-MSCs post thaw morphology on day [A.] 12th and [B.] 14th

However, each hGO NC significantly ($P<0.01$) decreased caprine WJ-MSCs post thaw viability in doses 100 and 50 $\mu\text{g/ml}$ as compared to 25, 10 and 0 $\mu\text{g/ml}$ doses which non-significantly differed (Table 3). Here, it showed that, each hGO NC in doses 25 and 10 $\mu\text{g/ml}$ vitrification solution not caused any significant cytotoxic effect on caprine WJ-MSCs post thaw viability.

Post thaw caprine WJ-MSCs were grew in colonies and observed elongated morphology typical as fibroblast cells and only minute cells were floated in media and detached from surfaces and observed confluent monolayer on days 14th (Figure 3).

4 Discussion

The aim of the present experiment was to study comparative effect of hGO NCs on caprine WJ-MSCs post thaw viability. Caprine WJ-MSCs were successfully isolated and cultured till third passage and phenotypically characterized by Alzarin red and Oil red O staining and confirmed their osteocytes and adipocytes differentiation capability, respectively (Figure 2). Caprine WJ-MSCs are fibroblast like cells which differentiated into osteocytes, adipocytes and chondrocytes (Somal et al., 2016) and cryopreservation did not alter their stemness but, post thaw cell viability was reduced ($< 60\%$) as like present study in control

Table 3 Effect of hGO NCs on caprine WJ-MSCs post thaw viability (n=3, $P<0.01$)

Sr. No.	hGO NCs	Post thaw cell viability % at different doses				
		100	50	25	10	0
1.	N ₂ -GO-HA NC	37.67 $\pm$ 2.40 ^a	47.67 $\pm$ 2.03 ^b	54.33 $\pm$ 3.71 ^{bc}	54.67 $\pm$ 1.45 ^{bc}	57.67 $\pm$ 2.03 ^c
2.	P-GO-HA NC	32.33 $\pm$ 2.96 ^a	38.00 $\pm$ 1.53 ^a	51.33 $\pm$ 3.38 ^b	50.67 $\pm$ 3.38 ^b	57.33 $\pm$ 2.73 ^b
3.	S-GO-HA NC	34.00 $\pm$ 2.08 ^a	42.67 $\pm$ 2.03 ^b	57.00 $\pm$ 1.15 ^c	55.33 $\pm$ 1.76 ^c	60.67 $\pm$ 1.45 ^c
4.	N ₂ -GO-SiO ₂ NC	39.67 $\pm$ 4.63 ^a	47.67 $\pm$ 2.96 ^{ab}	52.00 $\pm$ 3.79 ^{bc}	54.33 $\pm$ 1.86 ^{bc}	59.00 $\pm$ 1.73 ^c
5.	P-GO-SiO ₂ NC	40.00 $\pm$ 1.53 ^a	46.33 $\pm$ 0.88 ^b	55.67 $\pm$ 2.33 ^c	56.33 $\pm$ 1.45 ^c	58.33 $\pm$ 1.76 ^c
6.	S-GO-SiO ₂ NC	36.00 $\pm$ 2.89 ^a	45.67 $\pm$ 3.76 ^b	55.67 $\pm$ 2.03 ^c	54.00 $\pm$ 2.65 ^{bc}	56.33 $\pm$ 1.45 ^c
7.	N ₂ -GO-TiO ₂ NC	36.00 $\pm$ 1.73 ^a	40.67 $\pm$ 1.76 ^a	50.67 $\pm$ 2.60 ^c	50.33 $\pm$ 2.67 ^c	55.33 $\pm$ 2.03 ^c
8.	P-GO-TiO ₂ NC	35.67 $\pm$ 2.03 ^a	48.00 $\pm$ 2.65 ^b	56.33 $\pm$ 2.40 ^c	58.67 $\pm$ 2.03 ^c	59.00 $\pm$ 2.31 ^c
9.	S-GO-TiO ₂ NC	35.00 $\pm$ 3.79 ^a	39.00 $\pm$ 3.46 ^a	50.67 $\pm$ 1.45 ^b	52.00 $\pm$ 2.08 ^b	58.00 $\pm$ 2.65 ^b
10.	N ₂ -GO-Au NC	33.33 $\pm$ 2.60 ^a	38.33 $\pm$ 1.86 ^a	54.33 $\pm$ 2.73 ^b	49.00 $\pm$ 5.03 ^b	57.67 $\pm$ 2.96 ^b
11.	P-GO-Au NC	30.00 $\pm$ 3.06 ^a	39.00 $\pm$ 4.04 ^b	55.67 $\pm$ 1.45 ^c	52.67 $\pm$ 0.88 ^c	55.67 $\pm$ 1.45 ^c
12.	S-GO-Au NC	35.33 $\pm$ 3.84 ^a	35.33 $\pm$ 2.33 ^a	53.67 $\pm$ 2.03 ^b	56.33 $\pm$ 0.88 ^b	58.67 $\pm$ 1.20 ^b

Mean values bearing superscript in row differed significantly from each other.

group (Somal et al., 2017). However, hWJ-MSCs post thaw viability not significantly differed and observed $95 \pm 2.3\%$ survival rate and fibroblast like morphology (Massood et al., 2013) which is also justified in present study that, caprine WJ-MSCs post thaw morphology did not change and cells typically showed their fibroblast like morphology. In addition, post thaw rat BM-MSCs were formed colonies and showed fibroblastic or spindle shaped morphology and post thaw cell viability for vitrification method and OPS vitrification method observed as $81.33 \pm 6.93\%$ and $80.83 \pm 6.4\%$, respectively (Bahadori et al., 2009). GBNs such as graphene quantum dots (Dar et al., 2015), GO-Fe₂O₃ NCs (Gade et al., 2015) and N₂-GO-HA NCs (Dhenge et al., 2018) were exhibited dose (10, 25, 50 and 100 µg/ml) dependent cytotoxicity in caprine WJ-MSCs. However, nanoparticles incorporation in cryoprotectant solutions can improve potency of vitrification solution (Li et al., 2016) by changing its chemical properties (Xu et al., 2016) and this is justified in present study, as all hGO NCs in doses 10 and 25 µl/ml did not significantly ($P < 0.01$) reduced caprine WJ-MSCs post thaw viability as compared to control (Table 3). But, each hGO NCs in 50 and 100 µl/ml doses were significantly ($P < 0.01$) reduced caprine WJ-MSCs post thaw viability as compared to 0, 10 and 25 µl/ml doses. Hence, in present study it was confirmed that, each hGO NC in doses 10 and 25 µl/ml in vitrification solution did not cause any cytotoxicity in caprine WJ-MSCs during cryopreservation. Here, GO in each hGO NC reduced its cytotoxicity slightly which is in agreement with earlier findings as significantly ($P < 0.01$) increased human osteoblast viability and alkaline phosphatase activity (ALP) that cultured on HA-GN NCs powder deposited on Ti substrates (Liu et al., 2014). But, high GO concentration (0.5 wt. %) in film suppressed cell spreading and significantly inhibited both proliferation and ALP activity of MC3T3-E1 cells (Nishida et al., 2014). However, caprine BM-MSCs cultured on GO coated tissue culture plates exhibited fibroblast like typical MSCs morphology, grew in clusters (200-250), $>95\%$ viability with 24 hrs population doubling time and observed similar cellular proliferation in plane as well as GO coated tissue culture plates (Elkhenany et al., 2015) which might be justified in present study as GO absolutely enhanced biocompatibility of each hGO NC in 10 and 25 µg/ml doses by showing similar caprine WJ-MSCs post thaw viability (Table 3). However, cumulatively, significant ($P < 0.01$) lowest and highest caprine WJ-MSCs post thaw viability was observed in P-GO-HA NC and P-GO-TiO₂ NC incorporated groups, respectively and this might be due to HA (Baradaran et al., 2014) and TiO₂ nanoparticles (Salla et al., 2013) which significantly decreased and increased human osteoblast cell viability and MSCs proliferation, respectively than control. Hybridization of GN and GO with bio-mineral like HA increased mouse osteoblast (MC3T3-E1) cell viability (Kim et al., 2011) and also Au-GO entered in cytoplasm and increased enhanced Raman signals in HeLa cells with increased survivability and proliferation (Liu et al., 2012). In

addition, in support to present study, similarly GO and GO-SiO₂ nanoparticles significantly increased human neuronal stem cells (hNSCs) viability with highest expression of neuronal (TuJ1, MAP2 and Synapsin) and axonal (GAP43) markers (Solanki et al., 2013). Similarly, GO filler promotes proliferation in L929 and MG63 cell lines but concentration of GO coatings determined *in vitro* biocompatibility of Ti and HA nano sheets (Li et al., 2014). However, core shell fluorescently labelled 15, 60 and 200 nm size SiO₂ nanoparticles exhibited biocompatibility at doses 10, 20, 30, 40 and 50 µg/ml in THP-1 derived macrophages, A549 epithelial cells, HaCaT keratinocytes and NRK-52E kidney cells (Hsiao et al., 2014). In addition, ~50 nm, ~200 nm and ~400 nm size core-shell fluorescent SiO₂ nanoparticles did not decreased human MSCs viability at concentration 10, 50, 100 and 250 µg/ml (Yang et al., 2017). Also, TiO₂, SiO₂ and multi-walled carbon nanotubes (MWCNTs) were exhibited cytotoxicity in 3T3 fibroblast, RAW 264.7 macrophage and human bronchiolar epithelial cells (hT) in 100 and 1000 µg/ml and SiO₂ exhibited highest cytotoxicity in RAW cells (Sohaebuddin et al., 2010). These findings are not in agreement with the results of present study where N₂, P and S doped hybrid GOs with SiO₂ and TiO₂ nanoparticles significantly ($P < 0.01$) decreased caprine WJ-MSCs post thaw viability in 50 and 100 µg/ml doses (Table 3) and this difference in cell viability might be due to difference *in vitro* cell models. However, SiO₂ coated HA triphasic (Ca-66.36%, P-25.33% and Si-8.29%) biocomposite (HA-SiO₂) scaffolds (0.3x0.2x0.1 cm dimension) were showed biocompatibility in rabbit BM-MSCs (Ravindran et al., 2016) which is as supportive to present study findings in doses 10 and 25 µg/ml.

Conclusion

The present study concluded as, caprine WJ-MSCs are fibroblast like cells and differentiate into osteocytes and adipocytes. Post thaw caprine WJ-MSCs viability is decrease by hGO NCs at higher doses (100 and 50 µl/ml) and amongst all hGO NCs, P-GO-TiO₂ NC is least cytotoxic and it can be use at lower doses (25 and 10 µl/ml) in stem cell research.

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Conflicts of interest

None declared.

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