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Analysis of hybrid graphene oxide nanocomposites (hGO NCs) *in vitro* hemolytic impact in caprine erythrocytes

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Abstract
Graphene oxide (GO) is a carbon based nanomaterial extensively used in biosciences. In present study, comparative *in vitro* hemolytic effect of twelve hybrid GO nanocomposites (hGO NCs) (N_2-GO-HA, P-GO-HA, S-GO-HA, N_2-GO-SiO_2, P-GO-SiO_2, S-GO-SiO_2, N_2-GO-TiO_2, P-GO-TiO_2, S-GO-TiO_2, N_2-GO-Au, P-GO-Au and S-GO-Au) was assessed in caprine erythrocytes at 100, 50, 25, 10, and 0 µg/ml doses by hemolysis assay. In this study, it was observed that, significantly (P<0.01) highest (5.06±0.50%) and lowest (1.90±0.12%) hemolysis was occurred in caprine erythrocytes treated with S-GO-SiO_2 NC and P-GO-TiO_2 NC, respectively. Caprine erythrocytes were significantly (P<0.05) hemolysed by all twelve hGO NCs at 100 and 50 µg/ml doses as compared to 25, 10 and 0 µg/ml doses except S-GO-HA, N_2-GO-TiO_2, N_2-GO-Au, P-GO-Au and S-GO-Au where, only slight dose dependent hemolysis was observed. The present study is concluded that, S-GO-SiO_2 was highly hemolytic and P-GO-TiO_2 least hemolytic in caprine erythrocytes and however, all hGO NCs were cytocompatible at low doses (25 and 10 µg/ml) doses and hemolytic at high doses (100 and 50 µg/ml) in caprine erythrocytes.

Keywords: nanotechnology, graphene oxide, caprine erythrocytes

Introduction
Nanotechnology is the study of nano scale materials and recently, it is applied in chemical technology, environment, energy, information technology, biomedicine, agricultural as well as food industry. In biological sciences, the nanomaterials are used in drug discovery and delivery, bio-imaging and biosensors, tissue engineering and regenerative medicine, genomics and therapeutics [1] Graphene nanomaterial is an atom thick monolayer of carbon atoms arranged in a two dimensional honeycomb structure and is used in tissue engineering and other biological applications [2]. Graphene oxide (GO) is an oxygenated derivative of graphene and it has good biocompatibility [3] and heteroatoms doped GO and its nanocomposites exploits their properties [4]. Heteroatoms doped hybrid GO nanocomposites (hGO NCs) are as long as new tools in drug delivery, anticancer activity, markers, imaging, tissue engineering as well as in stem cell research [5]. In therapeutics and *in vivo* diagnostics, exogenously injected nanomaterials are transports in blood and are delivering drugs or other biologically active substances at specific tissues, act as contrast medium in bio-imaging techniques [6]. Exogenously injected nanomaterials in circulation remain very close with blood cells and therefore, it can disturb blood cells activity, metabolism alongwith alterations in blood cells size and results in circulatory diseases [7]. Prior to use novel hybrid nanomaterials, its details *in vitro* cytotoxicity analysis in different species models is most essential criterion. Because, the nanomaterials cytotoxicity is depends on size, dose and its types as well as *in vitro* cell models [8]. Caprine species is often used as pre-clinical *in vivo* [9] as well as *in vitro* [10] ruminant animal model to study human diseases. Hence, *in vitro* hGO NCs cytotoxicity assessment in animal models is pre-requisite and therefore, present study was conducted to assess comparative dose dependent *in vitro* hemolytic effect of twelve different hGO NCs in caprine erythrocytes.

Materials and Methods
Isolation of caprine erythrocytes and hemolysis assay: Experimental protocol was approved by Institution Animal Ethics Committee and study was undertaken at Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal

Husbandry, Anjora, Dist. Durg (C.G.). Caprine erythrocytes were isolated from fresh caprine blood and hemolysis assay [11] was carried out to analyse hGO NCs *in vitro* hemolytic activity in caprine erythrocytes at 100, 50, 25, 10 and 0 µg/ml (control) doses. Caprine erythrocytes were incubated in microcentrifuge tubes in triplicate with twelve different hGO NCs (N_2-GO-HA, P-GO-HA, S-GO-HA, N_2-GO-SiO_2, P-GO-SiO_2, S-GO-SiO_2, N_2-GO-TiO_2, P-GO-TiO_2, S-GO-TiO_2, N_2-GO-Au, P-GO-Au and S-GO-Au) as per indicated doses in Dulbecco's Phosphate Buffer Saline at 37^0C in incubator for 4 hrs. Sample tubes were shaken periodically and at the end of incubation period, samples tubes were centrifuged and haemoglobin in supernatant was transferred with due care in 96 wells flat bottom tissue culture plate. Haemoglobin absorbance was read thrice at 492 nm in ELISA plate reader and hemolysis % was calculated as per the following formula,

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

Statistical analysis: Results in the present study were analysed by Full Factorial Design and One way ANOVA (Post Hoc by Duncan test) using IBM SPSS Statistics 25 software. $P<0.01$ and $P<0.05$ values were considered statistically significant and results are presented as Mean ± S.E.

Results

In present study, comparatively significantly ($P<0.01$) highest hemolysis was occurred in caprine erythrocytes treated with S-GO-SiO_2 while, significantly ($P<0.01$) lowest hemolysis occurred in P-GO-TiO_2 treated caprine erythrocytes (Table 1). However, each hGO NC was significantly ($P<0.05$) hemolysed caprine erythrocytes at 100 and 50 µg/ml doses as compared to 25, 10 and 0 µg/ml doses except S-GO-HA, N_2-GO-TiO_2, N_2-GO-Au, P-GO-Au and S-GO-Au where, a little hemolysis was occurred as compared to control (Table 1).

Table 1: *In vitro* hemolytic effect of hGO NCs in caprine erythrocytes (Mean ± S.E.)

Sr. No.	hGO NCs	Hemolysis (%) at different doses (µg / ml)					*Cummulative hemolysis %
		100	50	25	10	0	
1.	N_2-GO-HA	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}	2.66 ± 0.25^{a}
2.	P-GO-HA	4.10 ± 0.83^{b}	4.07 ± 0.44^{b}	2.25 ± 0.52^{a}	1.66 ± 0.18^{a}	1.39 ± 0.11^{a}	2.69 ± 0.31^{a}
3.	S-GO-HA	3.53±0.82	2.75±0.70	3.91±0.46	1.98±0.69	1.33±0.19	2.70 ± 0.30^{a}
4.	N_2-GO-SiO_2	5.06 ± 0.71^{c}	2.81 ± 0.43^{b}	1.72 ± 0.35^{ab}	1.70 ± 0.24^{ab}	1.34 ± 0.10^{a}	2.52 ± 0.31^{a}
5.	P-GO-SiO_2	4.41 ± 1.00^{b}	5.42 ± 0.40^{b}	4.90 ± 1.12^{b}	4.32 ± 1.38^{b}	1.39 ± 0.17^{a}	4.09 ± 0.44^{b}
6.	S-GO-SiO_2	6.51 ± 0.17^{bc}	7.21 ± 1.31^{c}	5.90 ± 1.01^{bc}	4.33 ± 0.82^{b}	1.35 ± 0.03^{a}	5.06 ± 0.50^{c}
7.	N_2-GO-TiO_2	2.32 ± 0.27^{ab}	3.32 ± 0.66^{b}	2.56 ± 0.32^{ab}	1.64 ± 0.21^{a}	1.33 ± 0.13^{a}	2.23 ± 0.18^{a}
8.	P-GO-TiO_2	2.80 ± 0.52^{b}	1.91 ± 0.35^{ab}	1.74 ± 0.12^{a}	1.69 ± 0.03^{a}	1.34 ± 0.13^{a}	1.90 ± 0.12^{a}
9.	S-GO-TiO_2	3.84 ± 0.26^{c}	2.25 ± 0.42^{b}	1.83 ± 0.14^{ab}	1.72 ± 0.11^{ab}	1.36 ± 0.06^{a}	2.20 ± 0.20^{a}
10.	N_2-GO-Au	3.92±0.53	2.69±0.80	2.70±0.57	1.99±0.43	1.33±0.03	2.53 ± 0.26^{a}
11.	P-GO-Au	2.70±0.40	2.42±0.78	2.77±0.28	1.75±0.31	1.34±0.05	2.20 ± 0.18^{a}
12.	S-GO-Au	2.96±0.74	1.92±0.23	2.64±0.47	2.79±0.70	1.33±0.03	2.33 ± 0.20^{a}

Mean values bearing superscript in rows differed significantly from each other ($P<0.05$). *Mean values bearing superscript in column (cummulative hemolysis) differed significantly from each other ($P<0.01$).

Discussion

In this study, it was investigated as all hGO NCs are cytotoxic at high doses (100 and 50 µg/ml) and biocompatible at low doses (25 and 10 µg/ml) in caprine erythrocytes and comparatively, S-GO-SiO_2 was highly hemolytic while, P-GO-TiO_2 was least hemolytic in caprine erythrocytes. Blood cells are securely remains in plasma suspension and blood constituents as proteins and other organic or inorganic elements that bind with nanocomposites which form agglomeration [12] and might be reduced their influence on blood cells. In accordance with the present findings, earlier reported as isolated erythrocytes significantly hemolysed by Ag nanocomposites certainly at 100 µg/ml dose and no hemolysis was occurred at 1, 5, 10 and 50 µg/ml doses [13]. In support to this study, hybrid GOs (GO-H_2O, GO-PBS and GO-Manually grinding graphite) did not significantly reduce peripheral blood mononuclear cell viability at 2, 20 and 200 µg/ml doses [14]. However, as like present study findings, TiO_2 nanoparticles were significantly ($P<0.01$) reduced human blood cells values at doses 50, 250 and 500 µg/ml and washed erythrocytes were non significantly hemolysed at certain doses even at 500 µg/ml as compared to control after 3 hrs exposure [12]. In this study, hGO NCs cytotoxicity might be reduced by doped GO with organic nanomaterials which is previously justified as oxidized single walled carbon nanotubes (SWCNTs-OX) did not hemolysed erythrocytes at 20 nmol/L dose [15]. Also, similar with this study SiO_2 nanoparticles size dependent hemolysis was occurred in human erythrocytes at 50 and 100 µg/ml doses as compared with 10 and 20 µg/ml doses [16] however, pristine Ag nanoparticles at 10, 20 and 40 µg/ml doses significantly ($P<0.01$) hemolysed erythrocytes and decreased lymphocyte proliferation rate [17] unlike with present study findings.

Conclusion

It was concluded as, S-GO-SiO_2 was highly hemolytic and P-GO-TiO_2 was least hemolytic in caprine erythrocytes. However, all hGO NCs were cytocompatible at 25 and 10 µg/ml doses and hemolytic at 100 and 50 µg/ml doses in caprine erythrocytes and hGO NCs dose is considerable factor for determining hGO NCs biocompatibility in caprine erythrocytes.

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