The Pharma Innovation Journal 2020; 9(8): 127-135

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2020; 9(8): 127-135

www.thepharmajournal.com
Received: 14-06-2020
Accepted: 16-07-2020

Abinash Kumar
M.V.Sc Scholar, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science & A. H., CGKV, Anjora, Durg, Chhattisgarh, India

Nitin E Gade
Assistant Professor, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science & A. H., CGKV, Anjora, Durg, Chhattisgarh, India

OP Mishra
Head & Professor, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science & A. H., CGKV, Anjora, Durg, Chhattisgarh, India

GK Dutta
Professor, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science & A. H., CGKV, Anjora, Durg, Chhattisgarh, India

SA Dhenge
Assistant Professor, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science & A. H, Parbani, Maharashtra, India

SK Yadav
M.V.Sc Scholar, Department of Veterinary Surgery and Radiology, College of Veterinary Science & A. H., CGKV, Anjora, Durg, Chhattisgarh, India

Anupam Soni
M.V.Sc Scholar, Department of Livestock Production and Management College of Veterinary Science & A. H., CGKV, Anjora, Durg, Chhattisgarh, India

Shyamdeo Kumar
M.V.Sc Scholar, Department of Veterinary Surgery and Radiology, Bihar Veterinary College, Patna, Bihar, India

Corresponding Author:
Abinash Kumar
M.V.Sc Scholar, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science & A. H., CGKV, Anjora, Durg, Chhattisgarh, India

Effects of graphene quantum dots on physiological characteristics of Caprine spermatocytes

Abinash Kumar, Nitin E Gade, OP Mishra, GK Dutta, SA Dhenge, SK Yadav, Anupam Soni and Shyamdeo Kumar

Abstract
Present study was designed to evaluate the effects of different concentrations of graphene oxide quantum (GQ) dots on Caprine epididymal spermatocytes. Epididymal sperms were collected and incubated with different concentrations of GQ dots nanoparticles (0 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml). At the time of incubation and after 4hr, 8 hr and 12 hr semen quality parameters like live sperm count, sperm motility, acrosomal integrity, sperm abnormality and plasma membrane integrity was observed. Results showed that high concentrations (100 µg/ml) of GQ dots nanoparticles have harmful effects on sperm viability, acrosomal integrity and plasma membrane integrity. The effects of GQ dots were dependent on its concentration and duration of incubation. GQ dots have no significant effects on sperm abnormality and sperm motility. Plasma membrane integrity of spermatocytes is severely influenced by the GQ dots nanoparticles even at lower concentration (≥10 µg/ml). Significant decrease in live sperm count was observed in treatment groups (≥50 µg/ml) as compared to control. Acrosomal integrity was significantly affected 4 hr post exposure in treatment groups (≥50 µg/ml) however no change was observed after 8 hr & 12 hr exposure. Sperm abnormality was not affected in treatment groups. Plasma membrane integrity was significantly affected in treatment groups IV after 4 hr, 8 hr & 12 hr exposure to GQ dots.

Keywords: Quantam dots, caprine, spermatocytes, exposure, concentrations

1. Introduction
Nanotechnology deals with materials and structures ranging from 1 nm to 100 nm structural radius with specific physio-chemical properties. It has potential applications in the biomedical sciences such as drug therapy, diagnostics, tissue regeneration, cell culture and biosensors, cancer therapy, separation and purification of biological molecules and cells (Molday *et al*., 1982) [1]. Semen quality is proficiently affected with molecular defects in spermatozoa leading to reduced performance during artificial insemination. Organic flurophores are utilized as excellent tool for detection of damaged spermatozoa that target various functional parameters of the spermatozoa (Bussalleu *et al*., 2005) [2]. Spermatozoa selected by these molecular interactions progress into the tubal isthmus, and are trapped in its caudal portion, forming the oviduct sperm reservoir (Hunter, 1981) [3]. But the cost of fluorescence microscopy or flow cytometry limits their practicality in field (Sutovsky, 2015a) [4].
Potential applications of nanomaterials in reproductive biology are well reported and can be employed for field utility. Non-invasive manipulation of gametes with nanotechnology helps in improving the sperm fertility by reducing cryodamage and apoptopic and acrosome membrane damage during storage (Barranco *et al*., 2013; Lee *et al*., 2014) [5,6]. Novel approach of nanotechnology helps to investigate sperm function and fertility potential through *in vitro*-targeted labeling and purification as well as real-time *in vivo* imaging and tracking. Carbon nanotubes, carbon nanohorns, carbon nanofibers and graphene are mostly used in biomedical sciences especially in tissue engineering (Nishida, *et al*., 2016) [7]. Graphene oxide (GO) is oxygenated complement of Graphene. GO and metals, non-metals and organic nanomaterials were tested and used *in vitro* for biological applications individually in different models (Ghosh, *et al*., 2013; Kim, *et.al*., 2015; Figarol, *et al*., 2015) [8,9,10]. These nanomaterials are extensively investigated in a wide range of biomedical applications, in particular regenerative medicine and tissue engineering. Remarkable applications of these nanomaterials will attract industries for their production however their biological effects need to be studied (Ostrowski *et al*., 2009, Wani *et al*., 2011) [11, 12].

2. Material and method

Present study was conducted in the Veterinary Physiology and Biochemistry Department of College of Veterinary Science and A.H., Anjora, Durg, Chhattisgarh. The study was initiated in month of April with proper arrangement of all instruments which were required during experimental procedures and proper hygienic environment was maintained in the laboratory.

2.1. Media and Chemicals

Different media are used including Tissue culture medium (TCM 199), Dulbecco's phosphate buffer solution (DPBS), fetal bovine serum (FBS), antimycotic and antibiotic agent, mineral oil, Brilliant cresyl blue staining (BCB). Chemical which used it includes sodium chloride (NaCl), potassium chloride (KCl), calcium chloride dihydrate ($CaCl_2.2H_2O$), sodium citrate, sodium bicarbonate, HEPES, Pyruvic acid, Eosin and Nigrosine stain.

Experimental Design: Total three groups with different concentrations of GQ dots (10 µg/ml, 50µg/ml and 100µg/ml) were prepared to evaluate their cytotoxic effects on Caparine spermatocytes and compared with control (0 µg/ml). Sperms were incubated *in vitro* for 4 hr, 8 hr and 12 hr at 37° C in Tyrode's albumin lactate pyruvate (TALP) medium (Kharche *et al*., 2011) [13] with different concentrations GQ dots. Effects of GQ dots on sperm viability, motility, abnormality, acrosomal integrity and plasma membrane integrity were evaluated.

2.2. Parameters studied

- **Live dead count:** Live and dead count of sperm cells count take a drop of sperm sample was mixed with a drop of nigrosine-eosin stain on a clean slide, and the mixture was allowed to stand for 3 min. Then, the smear was air dried and examined under the microscope (100X magnifications). Dead spermatozoa were stained either partially or completely pink and live spermatozoa appeared colourless. 200 spermatozoa were randomly examined, and the percentages of live sperm cells were determined. The mean results were expressed as percent viable spermatozoa.
- **Sperm motility:** For sperm motility we were take a drop (100 µl) of sperm sample and placed on a prewarmed, grease-free slide. A cover slip was put over the 27 drop and examined under the microscope (40X magnification), and the percentage of sperm motility will be determined.
- **Acrosomal integrity:** For acrosomal integrity, a small drop of each sample will be placed on a clean slide and a smear was made. The air-dried smears were fixed in Hancock's solution for 15-20 min, washed and rinsed with distilled water. The slides were stained with Giemsa working solution overnight, then rinsed with tap water, air-dried and observed under the microscope (100X magnification). 200 spermatozoa were examined, and the percentage of intact acrosome will be determined (Watson *et al*., 1973) [14].
- **Sperm abnormality:** For sperm abnormality, mix one drop of semen with eight drops of the nigrosine-eosin stain and incubate the mixture at 37°C. Prepare the smear and examine the morphology of sperm microscopically at 40X and 100X magnification.
- **Plasma membrane integrity by Hypo-osmotic swelling test (HOST):** For this test 100 µl semen and 1ml HOST solution was mixed gently with the pipette and kept at 37°C for 30 minutes. 200 spermatozoa were observed at 40X magnification for Hypo-osmotic swelling. Live sperm cells were distinguished by swelling and curling of the sperm tail; sperms with curled tails were live spermatozoa.

2.3. Stastical analysis

Experimental data in this study were expressed as Mean ± SE and analyzed by one way and two-way analysis of variance (ANOVA) by using SPSS software described by Snedecor and Cochran (1989) [15].

3. Result and discussion

Effects of different concentrations of GQ dots on Caprine sperm: Epididymal sperms harvested from testes and incubated for 12 hr at 37 °C in different concentrations of GQ dots in TALP. The effects on various sperm quality parameters were recorded at 0 hr, 4 hr, 8 hr and 12 hr intervals.

3.1. Live dead count

Effects on sperm viability at different concentrations of GQ dots nanoparticle were assessed by determining and comparing live sperm count (%) at different interval (0 hr, 4 hr, 8 hr and 12 hr). Results showed that mean (± S.E) percentages of live sperm count at 0 hr were 74.00 ± 1.15 (Group I), 70.67 ± 1.96 (Group II), 69.67 ± 1.45 (Group III), 71.50 ± 0.76 (Group IV). There was no significant difference in percentages of live sperm count in all treatment groups and control. After 4 hr of incubation mean (± S.E) percentages of live sperm count were 49.00 ± 0.58 (Group I), 47.67 ± 2.19 (Group II), 44.17 ± 1.42 (Group III) and 37.83 ± 0.73 (Group 31 IV). Group IV showed lowest percentage of live sperms and Group IV and III differed significantly from control group however group II and control group have comparable live sperm counts. After 8 hr of incubation mean (± S.E) percentages of live sperm count were 36.00 ± 1.73 (Group I), 25.67 ± 1.20 (Group II), 27.67 ± 2.20 (Group III), 26.67 ± 4.18 (Group IV). Group II and IV showed lower percentage of live sperms and it differed significantly from control after 8 hr exposure. Group III has comparable viable sperm cells count with group II, IV and control. After 12 hr of incubation mean (± S.E) percentages of live sperm count were 12.67 ± 1.09 (Group I), 10.33 ± 1.20 (Group II), 10.83 ± 0.93(Group III) and 9.17 ± 1.64 (Group IV), (Table. 1). There was no significant difference among all treatment and control groups after 12 hr exposure. Significant variations in percentages of live sperm cells were observed within all treatment groups and control at 0 hr, 4 hr, 8 hr and 12 hr exposure.

Table 1: Effects of GQ dots NPs on live dead count of sperm of different groups at different intervals (Mean ± S.E)

Interval/ Group	Live sperms (%)			
	I	II	III	IV
0 hr.	74.00 ± 1.15^{a}	70.67 ± 1.96^{a}	69.67 ± 1.45^{a}	71.50 ± 0.76^{a}
4 hrs.	49.00 ± 0.58^{bx}	47.67 ± 2.19^{bxy}	44.17 ± 1.42^{by}	37.83 ± 0.73^{bz}
8 hrs.	36.00 ± 1.73^{cx}	25.67 ± 1.20^{cy}	27.67 ± 2.20^{cxy}	26.67 ± 4.18^{cy}
12 hrs.	12.67 ± 1.09^{d}	10.33 ± 1.20^{d}	10.83 ± 0.93^{d}	9.17 ± 1.64^{d}

abcd value bearing superscripts in column and xyz bearing superscripts in row differ significantly from each other ($p<0.05$)

Sperm viability is pre-requisite because only live progressively motile sperms can contribute to fertilization in female reproductive tract. Therefore, the live sperm count is an important quality parameter of sperm function test. Results

show significant decline in viable sperm count after incubation with higher concentration of GQ dots (100µg/ml) (Fig.1 & 2). Some of previous reports are similar to our observation, Barkhordari *et al.*, (2013) [16] observed that the survivability of human sperm was decreased with increasing time interval and exposure of high concentration of ZnO NPs (upon 1000 µg/ml). Percentage of sperm mortality was 20.8%, 21.2% and 33.2% at 45 min, 90 min and 180 minutes respectively.

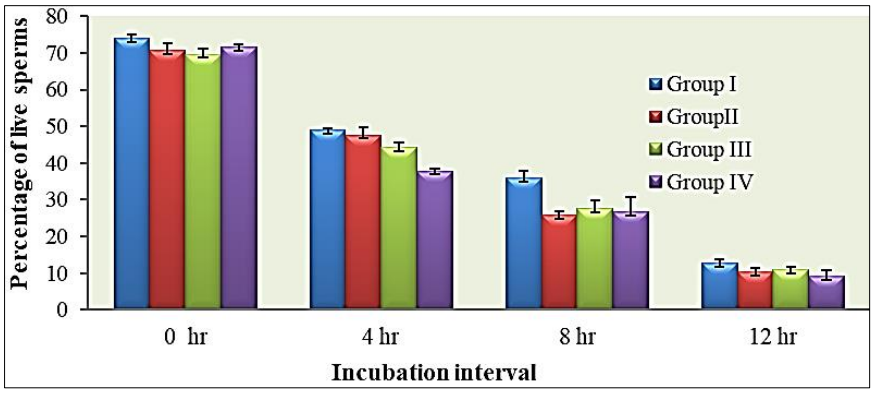
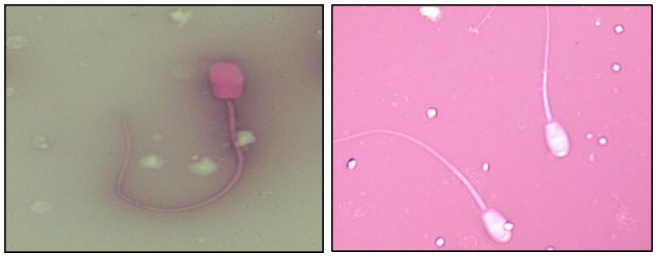


Fig 1: Effects of different concentrations GQ dots on Caprine live sperm count (%)



A: Live sperm **B: Dead sperm**

Fig 2: Nigrosine-Eosin staining for live dead count

Wang *et al.*, (2013) [17] reported that after intra-articular injection of CoCr NPs in male rats and high dose of CoCr NPs could significantly decrease the percentage of live sperm count. Wisniewski *et al.*, (2015) [18] reported that percentage of live sperm cell significantly decrease in BPA- treated rat. Asghar *et al.*, (2016) [19] reported that up to 25 µg/ml concentration of reduced graphene oxide have no effects on human sperm after 30 min and 3 hr incubation. Similar results were reported in mouse sperms after repeated exposure to carbon nanotubes (Bai *et al.*, 2010) [20]. Our results are in accordance with previous studies where low concentration exposure for short duration has no effects on sperm viability. However increasing concentration and duration of exposure have detrimental effects on live sperms. Chandra *et al.*, (2007) [21] reported that sperm viability is lower in Cr treated animal in comparison to untreated animal. Pothuraju *et al.*, (2014) [22] reported that after treatment with different concentration of Ag- NPs buffalo spermatozoa, the viability of buffalo spermatozoa was decreased with increased concentrations of Ag NPs. Gold nanoparticles have no significant effect on sperm viability in bovine spermatocytes (Taylor *et al.*, 2014) [23]. In contrast, Aporvari *et al.*, (2018) [24] reported that percentage of sperm viability of Arabic ram sperm increases in treated group with dietary ZnO nanoparticles as compared to control.

3.2. Sperm Motility

Effects of different concentration of GQ dots nanoparticle on sperm motility was assessed by determining and comparing sperm motility percentage at different interval (0 hr, 4 hr, 8 hr and 12 hr). Results showed that mean (± S.E) percentages of sperm motility at 0 hr were 74.33 ± 1.20 (Group I), 75.33 ± 1.20 (Group II), 74.67 ± 1.45 (Group III) and 75.34 ± 0.88 (Group IV). There was no significant difference in sperm motility in all treatment groups and control at 0 hr. After 4 hr of incubation mean (± S.E) percentages of sperm motility were 54.67 ± 1.76 (Group I), 53.67 ± 1.45 (Group II), 55.67 ± 1.86 (Group III) and 52.33 ± 1.20 (Group IV). Group IV have lowest motility however there was no significant difference in sperm motility in all treatment groups and control at 4 hr. After 8 hr of incubation mean (± S.E) percentages of sperm motility were 23 ± 2.08 (Group I), 23 ± 1.15 (Group II), 24.33 ± 0.88 (Group III) and 22.67 ± 1.20 (Group IV). Group IV have lowest motility however there was no significant difference in sperm motility in all treatment groups and control at 8 hr. After 12 hours of incubation mean (± S.E) percentages of sperm motility were 8.33 ± 1.20 (Group I), 6.67 ± 0.88 (Group II), 6.67 ± 1.45 (Group III) and 6.33 ± 0.88 (Group IV) (Table 2) Group IV have lowest motility however there was no significant difference in sperm motility in all treatment groups and control at 12 hr.

Table 2: Effects of GQ dots NPs on sperm motility of different groups at different intervals (Mean ± S.E)

Interval/ Group	Sperms motility (%)			
	I	II	III	IV
0 hr.	74.33 ± 1.20^{a}	75.33 ± 1.20^{a}	74.67 ± 1.45^{a}	75.34 ± 0.88^{a}
4 hrs.	54.67 ± 1.76^{b}	53.67 ± 1.45^{b}	55.67 ± 1.86^{b}	52.33 ± 1.20^{b}
8 hrs.	23.00 ± 2.08^{c}	23.00 ± 1.15^{c}	24.33 ± 0.88^{c}	22.67 ± 1.20^{c}
12 hrs.	8.33 ± 1.20^{d}	6.67 ± 0.88^{d}	6.67 ± 1.45^{d}	6.33 ± 0.88^{d}

abcd value bearing superscripts in column ($p< 0.05$)

Significant variation in percentages of sperm motility was observed within all treatment groups and control at 0 hr, 4 hr, 8 hr and 12 hr incubation. Sperm motility is an indicator of male fertility and progressive movement sperm is directly affecting their headway in female reproductive tract. Any change in sperm motility is having direct impact on fertility

(Fig.3). Present study reports non-significant decline in sperm motility after incubation with higher concentration of GQ dots. Previous reports have variable results with different concentrations and types of nonmaterial's. Ram semen supplemented with CeO2 nanoparticles incubated for 24 hrs at 40 C showed unaffected sperm motility (Falchi *et al.,* 2016) [25]. In bovine semen also sperm motility remains unchanged when it was exposed to iron oxide (Fe3O4-NPs) and europium hydroxide conjugated with polyvinyl alcohol or polyvinylpyrrolidone (Makhluf *et al.,* 2008, Makhluf *et al.,* 2006) [26, 27].

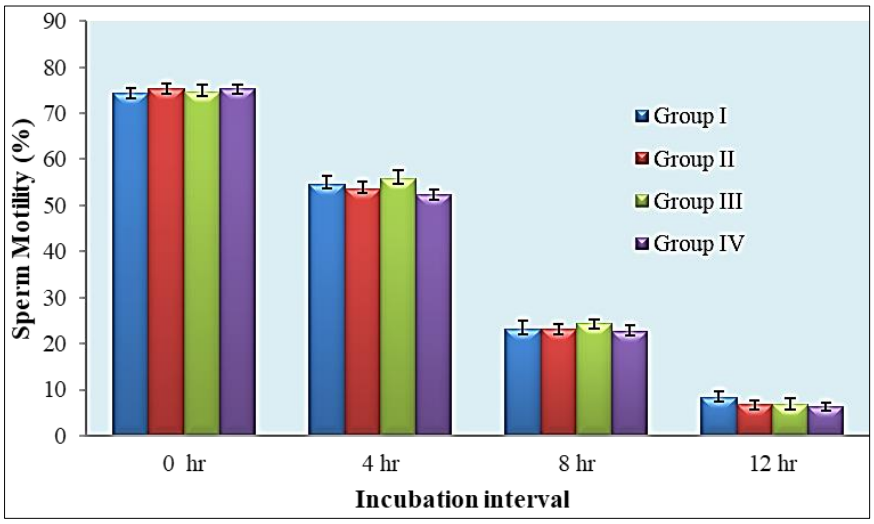


Fig 3: Effects of different concentrations GQ dots on Caprine Sperm Motility (%)

Skovmand *et al.,* (2018) [28] reported that after pulmonary exposure of carbonaceous nanomaterials, there was no significant difference in sperm motility between the groups. Some previous reports are contrast to our observation, Torabi *et al.,* (2017) [29] observed that cyclophosphamide (CP) in male rat caused a significant decrease in sperm motility. Similarly, in bovine semen exposed to gold nanoparticles showed significant reduction in sperm motility (Taylor *et al.,* 2012) [30]. Apovari *et al.,* (2018) [24] reported that after oral administration of different quantity (40 ppm and 80 ppm) Zinc oxide NPs in Arabic ram's sperm motility was increased compared with the control group (p<0.05). It can be concluded that different nanoparticles have variable effects on sperm motility and increasing concentrations of GQ dots reduces sperm motility non-significantly after post exposure.

3.3. Acrosomal integrity

Effect of different concentration of GQ dots nanoparticle on percentages of sperm with intact acrosome were assessed by determining and comparing percentage of sperm with intact acrosome at different interval (0 hr, 4 hr,8 hr and 12 hr). Results showed that mean (± S.E) percentages of sperm with intact acrosome at 0 hr were 94 ± 0.58 (Group I), 91.33 ± 0.88 (Group II), 90.67 ± 1.20 (Group III) and 90.34 ± 1.86 (Group IV). There was no significant difference in acrosomal integrity in all treatment groups and control at 0 hr. After 4 hr of incubation mean (± S.E) percentages of sperm with intact acrosome were 92 ± 0.58 (Group I), 90.33 ± 0.33 (Group II), 87.67 ± 0.88 (Group III) and 84.67 ± 1.45 (Group IV). Group IV showed least acrosomal integrity and Group IV and Group III significantly differed from control group. When Group II and control group were compared no significant change in acrosomal integrity was observed at 4 hr. After 8 hr of exposure mean (± S.E) percentages of sperm with intact acrosome were 89 ± 0.58 (Group I), 89 ± 1.15 (Group II), 85.33 ± 0.88 (Group III) and 85.33 ± 1.86 (Group IV). There was no significant difference in acrosomal integrity in all treatment groups and control at 8 hr. After 12 hr of incubation mean (± S.E) percentages of sperm with intact acrosome were 85.33 ± 0.67 (Group I), 85 ± 1.15 (Group II), 84.67 ± 0.67 (Group III), 85.33 ± 0.88 (Group IV) (Table 3). There was no significant difference in acrosomal integrity in all treatment groups and control at 12 hr.

Table 3: Effects of GQ dots nanoparticle on percentage of sperm with intact acrosome of different groups at different intervals (Mean ± S.E)

Interval/Group	Sperms with intact acrosome (%)			
	I	II	III	IV
0 hr.	94.00 ± 0.58^{a}	91.33 ± 0.88^{a}	90.67 ± 1.20^{a}	90.34 ± 1.86^{a}
4 hrs.	92.00 ± 0.58^{bx}	90.33 ± 0.33^{axy}	87.67 ± 0.88^{aby}	84.67 ± 1.45^{z}
8 hrs.	89.00 ± 0.58^{c}	89.00 ± 1.15^{a}	85.33 ± 0.88^{b}	85.33 ± 1.86
12 hrs.	85.33 ± 0.67^{d}	85.00 ± 1.15^{b}	84.67 ± 0.67^{b}	85.33 ± 0.88

abcd value bearing superscripts in row and xyz bearing superscripts in column differ significantly from each other. $p < 0.01$ (highly significant).

Within group I significant variation in percentage of sperm with intact acrosome was observed at 0 hr, 4 hr, 8 hr and 12 hr incubation. In group II, percentage of acrosomal integrity after incubation for 0 hr, 4 hr and 8 hr differed significantly from 12 hr of incubation. In group II, percentage of acrosomal integrity after incubation for 0 hr and 4 hr differed significantly from 8 hr and 12 hr of incubation. In group IV there was no significant difference in acrosomal integrity in all incubation intervals (0 hr, 4 hr, 8 hr and 12 hr) (Fig.4 & 5).

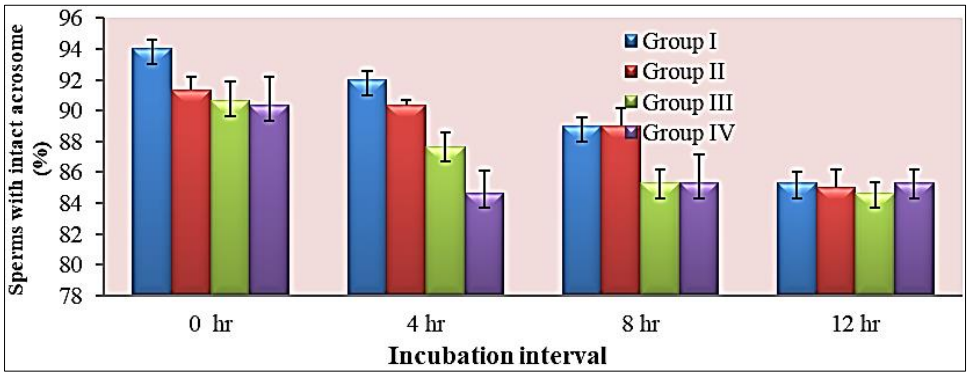
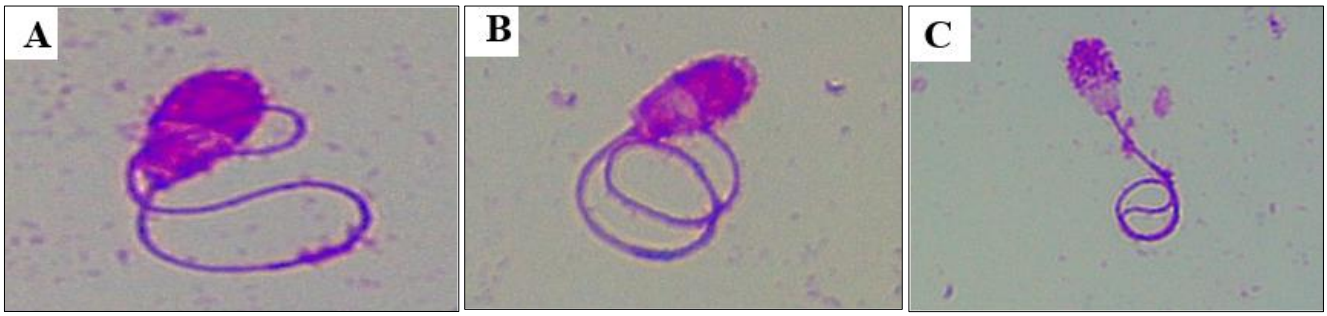


Fig 4: Effects of different concentrations GQ dots on acrosomal integrity of Caprine spermatocytes



Intact acrosome **Partially damaged acrosome** **Degraded acrosome**

Fig 5: Giemsa staining for acrosomal integrity

Acrosomal integrity is critical parameter in semen quality evaluation because structural changes in acrosome may affect the process of capacitation and ultimately fertilization. Intact acrosome is pre-requisite for capacitation and it precisely regulates fusion of oocyte and sperm. Results showed that co-incubation of sperms with high concentration of GQ dots for short duration (4 hr) significantly affected the acrosomal integrity but in long duration (8hr and 12hr) the effects subsided. Previous report showed that AuNPs have significant detrimental effects on the acrosomal integrity (Taylor *et al*., 2014) [23]. However some reports indicate that there is no significant effect of co-incubation with gold and silver (Barklina *et al.*, 2014) [31], mesoporus silica (Tiedemann *et al.*, 2014) [23], cadmium selenium and zinc sulphide quantum dots (Feugang *et al.*, 2012) [33] on acrosomal integrity. Similarly, in ram semen CeO2 have no significant effect on acrosomal integrity after 24 hr exposure (Falchi *et al.*, 2016) [25]. In Caprine and canines incubation intervals have significant effect on acrosomal integrity and it decreases probable outcome of fertilization (Swain *et al.*, 2012, Tarai *et al.*, 2010) [34, 35]. It concludes that GQ dots have short term effects on acrosomal integrity at higher doses which may affect the fertilizing ability of sperms.

3.4. Sperm abnormality

Effect of different concentration of GQ dots nanoparticle on sperm abnormality was assessed by determining and comparing sperm abnormal sperm percentage at different interval (0 hr, 4 hr, 8 hr and 12 hr). Results showed that mean (± S.E) percentages of sperm abnormality at 0 hr were 16.33 ± 1.33 (Group I), 17.67 ± 0.33 (Group II), 12.67 ± 2.33(Group III), 14.67 ± 2.73(Group IV). After 4 hr of incubation mean (± S.E) percentages of sperm abnormality were 15 ± 2.52 (Group I), 13.33 ± 0.88 (Group II), 17.33 ± 1.67 (Group III), 16.00 ± 1.53 (Group IV). After 8 hr of incubation mean (± S.E) percentages of sperm abnormality were 16 ± 2.31 (Group I), 13.67 ± 2.73 (Group II), 13.33 ± 0.88 (Group III), 17.67 ± 0.88 (Group IV). After 12 hr of incubation mean (± S.E) percentages of sperm abnormality were 15 ± 2.08 (Group I), 15.67 ± 1.76 (Group II), 17.33 ± 1.45 (Group III), 14.33 ± 1.86 (Group IV) (Table 4). There was no significant difference between and within all treatment groups and control in percentage of sperm abnormality at 0 hr, 4 hr, 8 hr and 12 hr incubation.

Table 4: Effects of GQ dots nanoparticle on percentage of sperm abnormality of different groups at different intervals (Mean ± S.E)

Interval/ Group	Sperm abnormality (%)			
	I	II	III	IV
0 hr.	16.33 ± 1.33	17.67 ± 0.33	12.67± 2.33	14.67 ± 2.73
4 hrs.	15.00 ± 2.52	13.33 ± 0.88	17.33 ± 1.67	16.00 ± 1.53
8 hrs.	16.00 ± 2.31	13.67 ± 2.73	13.33± 0.88	17.67 ± 0.88
12 hrs.	15.00 ± 2.08	15.67 ± 1.76	17.33 ± 1.45	14.33 ± 1.86

abcd value bearing superscripts in column and xyz bearing superscripts in row differ significantly from each other ($p< 0.05$)

Morphological abnormality of spermatocytes compromises its motility and fertility therefore it's an important sperm function test. Results show no significant difference in sperm abnormality after incubation with different concentration of GQ dots (Fig. 6 & 7). Some of previous reports are in accordance to our results, Taylor *et al*., (2010) [36] reported that in bovine sperm Au nanoparticle has no significant effect on sperm abnormality. Majority of morphological abnormalities

arises during spermatogenesis and spermiogenesis therefore post spermiation *in vitro* incubation has limited effects on sperm morphology.

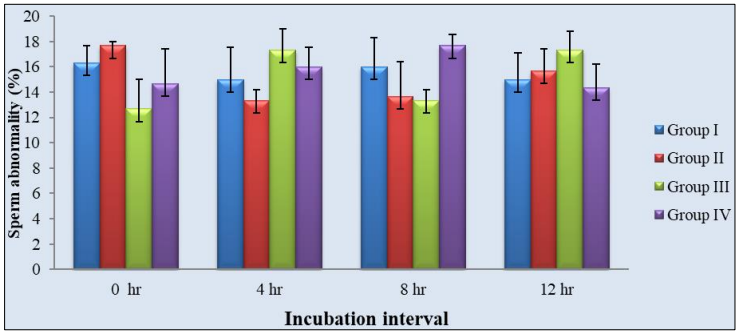


Fig 6: Effects of different concentrations GQ dots on Caprine Sperm abnormality

Head Mid-piece Tail

Fig 7: Nigrosine-Eosin staining for sperm abnormality

In vivo studies reported that after intra-articular injection of high dose of CoCr NPs in male rats significantly increase the abnormality of sperm (Wang *et al.*, 2013) [17] and similar result were observed by Wisniewski *et al.*, (2015) [18] in BPA treated rat. Nazar *et al.*, (2016) [37] observed that in treatment of male bulb-c mice with gold nanoparticles causes a significant increase in sperm abnormality. Wang *et al.*, (2013) [17] reported that after intra-articular injection of CoCr NPs in male rats and high dose of CoCr NPs could significantly increase the sperm abnormality. Abbasalipourkabir *et al.*, (2015) [38] observed that the sperm abnormality of male Wistar rat was decreased with increasing the concentration of ZnO nanoparticles.

3.5. Plasma membrane integrity by Hypo-osmotic swelling test (HOST)

Table 5: Effects of GQ dots nanoparticle on percentage of plasma membrane integrity of sperm of different groups at different intervals (Mean ± S.E)

Interval/Group	Reacted sperm (%) (HOST)			
	I	II	III	IV
0 hr.	68.33 ± 1.45^{a}	68.67 ± 2.33^{a}	65.33 ± 0.88^{a}	67.67 ± 1.45^{a}
4 hrs.	45.33 ± 0.88^{bx}	44.33 ± 1.86^{bx}	41.33 ± 1.86^{bx}	34.67 ± 0.88^{by}
8 hrs.	32.67 ± 2.03^{cx}	22.33 ± 0.88^{cy}	24.00 ± 2.08^{cy}	22.33 ± 3.84^{cy}
12 hrs.	9.33 ± 1.20^{dx}	6.33 ± 0.88^{xy}	9.33 ± 1.76^{dx}	4.00 ± 0.58^{dy}

abcd value bearing superscripts in column and xyz bearing superscripts in row differ significantly from each other ($p< 0.05$)

Effects of different concentrations of GQ dots nanoparticle on plasma membrane integrity were assessed by determining and comparing percentage of reacted sperm in HOST at different interval (0 hr, 4 hr, 8 hr and 12 hr). Results showed that mean (± S.E) percentages of strong reacted sperms at 0 hr were 68.33 ± 1.45 (Group I), 68.67 ± 2.33 (Group II), 65.33 ± 0.88 (Group III) and 67.67 ± 1.45 (Group IV). There was no significant difference in plasma membrane integrity in all treatment groups and control at 0 hr. After 4 hr of incubation mean (± S.E) percentages of strong reacted sperms were 45.33 ± 0.88 (Group I), 44.33 ± 1.86 (Group II), 41.33 ± 1.86 (Group III) and 34.67 ± 0.88 (Group IV). Group IV showed lowest percent of strong reacted sperms and plasma membrane integrity differed significantly from Group II, III and control group at 4 hr. After 8 hr of incubation mean (± S.E) percentages of strong reacted sperms were 32.67 ± 2.03 (Group I), 22.33 ± 0.88 (Group II), 24.00 ± 2.08 (Group III), 22.33 ± 3.84 (Group IV). Group II, III and IV differed significantly from control group at 8 hr. After 12 hr of incubation mean (± S.E) percentages of strong reacted sperms were 9.33 ± 1.20 (Group I), 6.33 ± 0.88 (Group II), 9.33 ± 1.76 (Group III), 4 ± 0.58 (Group IV). Group IV showed lowest percent of strong reacted sperms and it differed significantly from group III and control group at 12 hr. Significant variations in plasma membrane integrity were observed within all treatment groups and control at 0 hr, 4 hr, 8 hr and 12 hr incubation (Fig. 8 & 9).

HOST test is simple, cost effective technique to evaluate the integrity of plasma membrane (Vasquez *et al.*, 2013) [39]. In all

intervals (4 hr, 8 hr and 12 hr) higher concentrations of GQ dots have significant effect on plasma membrane integrity. Wisniewski *et al.*, (2015) [18] reported that the integrity of the plasma membrane was decreased by 2% in both BPA-treated groups. Mathias *et al*., (2015) [40] reported that after male Wistar rats were treated with 15-30 µg Ag NPs then there is significantly decrease in plasma membrane integrity. Pothuraju *et al.*, (2014) [41] reported that after treatment with different concentration of Ag- NPs buffalo bull spermatozoa, the plasma membrane integrity of spermatozoa was decreased with increased concentrations of Ag NPs. However, in contrast CeO2 nanoparticles have no significant effect on plasma membrane structures of ram semen after 24 hr exposure (Falchi *et al.*, 2016), Aporvari *et al.*, (2018) [25,24] reported that percentage of reacted sperms of Arabic ram sperm increases with the higher concentration of dietary ZnO nanoparticles. Omu *et al*., (1998) [42] observed that after treatment of asthenozoosperia with supplementation of Zn nanoparticle have beneficial effects on plasma membrane integrity.

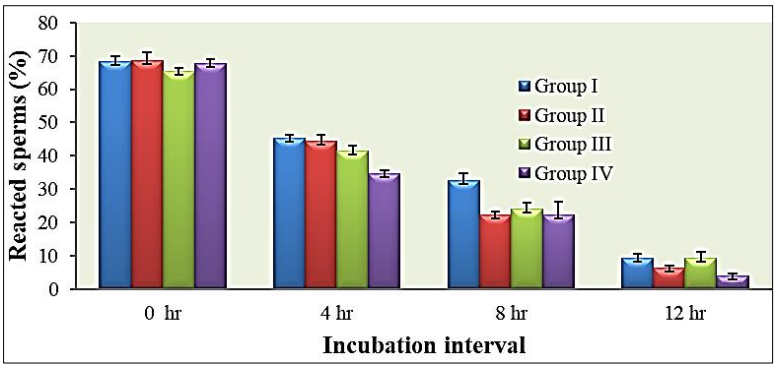
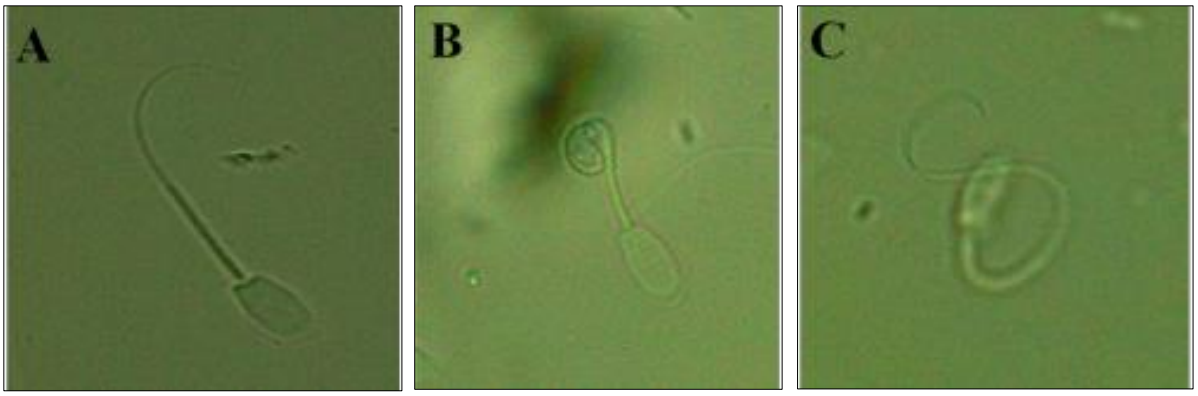


Fig 8: Effects of different concentrations GQ dots on Plasma Membrane

Integrity of caprine spermatocytes



Non-reacted **Moderate degree of tail coiling** **Strongly reacted with a severe tail coiling**

Fig 9: Hypo-osmotic swelling test for plasma membrane integrity

4. Conclusion

It can be concluded that high concentrations of GQ dots nanoparticles have harmful effects on sperm viability, acrosomal integrity and plasma membrane integrity. Plasma membrane integrity of spermatocytes is severely influenced by the GQ dots nanoparticles even at lower concentration (≥10µg/ml).

5. Acknowledgments

The authors are responsible for all the content.

6. Conflict of interest

The authors declare that they have no conflict of interest.

7. Funding information

This work was supported by college of veterinary science and animal husbandry, Anjora, Durg, Chhattisgarh, 491001 India.

8. References

1. Molday RS, Mackenzie D. Immunospecific ferromagnetic iron dextran reagents for the lebeling and magnetic separation of cells, J Immunol Methods. 1982; 52:353-367.
2. Bussalleu E, Pinart E, Yeste M, Briz M, Sancho S, Garcia-Gil N *et al*. Development of a protocol for multiple staining with 4 fluorochromes to assess the functional status of boar spermatozoa. Microscopy Research and Technique. 2005; 68(5):277-283.
3. Hunter RHF. Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. Journal of Reproduction and Fertility. 1981; 63(1):109-117.
4. Sutovsky P. New approaches to boar semen evaluation, processing and improvement. Reprod. Domes. Ani. 2015a; 50:11-19.
5. Barranco I, Ortega MD, Martinez-Alborcia MJ, Vazquez JM, Martinez EA, Roca J. Season of ejaculate collection influences the freezability of boar spermatozoa. Cryobiology. 2013; 67(3):299-304.
6. Lee WY, Lee R, Kim HC, Lee KH, Cui XS, Kim NH *et al*. Pig spermatozoa defect in acrosome formation caused

poor motion parameters and fertilization failure through artificial insemination and *In vitro* fertilization. Asian-Australasian Journal of Animal Sciences. 2014; 27(10):1417-1425.
7. Nishida E, Miyaji H, Takita H, Kanayama I, Tsuji M, Akasaka T *et al*. Graphene oxide 17 scaffold accelerates cellular proliferative response and alveolar bone healing of tooth extraction socket. Int. J. Nanomed. 2016; 11:2265-2277.
8. Ghosh M, Chakraborty A, Mukherjee A. Cytotoxic, genotoxic and the haemolytic effect of titanium dioxide (TiO2) nanoparticles on human erythrocyte and lymphocyte cell *in vitro*. J. Appl. Toxico. 2013; 33(10):1097-1110.
9. Kim HT, Waleed AES, Choi JW. Graphene based materials for stem cell applications. Materials. 2015; 8:8674-8690.
10. Figarol A, Jeremie P, Delphine B, Valerie F, Celine A, Jean MT *et al*. *In vitro* toxicity of carbon nanotubes, nanographite and carbon black, similar impacts of acid functionalization. J. Toxicol. *In Vitro*. 2015; 30(1):476-485.
11. Ostrowski A, Martin T, Conti J, Hurt I. and Harthorn B. Nanotoxicology: characterizing the scientific literature, J. Nanopart. Res. 2009; 11(2):251-257.
12. Wani M, Hashim M, Nabi F, Malik M. Nanotoxicity: Dimensional and Morphological Concerns Adv. in Phys. Chem. 2011; 15:450-912.
13. Kharche SD, Goel AK, Jindal SK, Goel P, Jha BK. Birth of twin kids following transfer of in-vitro produced caprine embryos. Indian J. Anim. Sci. 2011; 81:1132-1134.
14. Watson PE. Use of a Giemsa stain to detect changes in acrosome of frozen ram spermatozoa. Vet. Rec. 1975; 97:12-15.
15. Snedecor GW, Cochran WG. Statistical Methods, 8th ed. Oxford and IBH Publishing Company, New Delhi, 1989.
16. Barkhordari A, Hekmatimoghaddam S, Jebali A, Khalili MA, Talebi A, Noorani M. Effect of zinc oxide nanoparticles on viability of human spermatozoa Iran J Reprod Med. 2013; 11(9):767-771.
17. Wang T, Zhao G, Liang XM, Xu Y, Li Y, Tang H *et al*. Numerical simulation of the effect of super paramagnetic nanoparticles on microwave rewarming of cryopreserved tissues. Cryobiology, 2014; 68:234-243
18. Wisniewski P, Romano RM, Kizys MM, Oliveira KC, Kasamatsu T, Giannocco G *et al*. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic–pituitary–testicular axis. Toxicology. 2015; 329:1-9.
19. Asghar W, Shafiee H, Velasco V, Vasu R, Guo SS, Rami Assal E *et al*. Toxicology Study of Single-walled Carbon Nanotubes and Reduced Graphene Oxide in Human Sperm. Scientific Reports. 2016; 6:30270.
20. Bai Y, Zhang Y, Zhang J, Mu Q, Zhang W, Butch ER *et al*. Repeated administrations of carbon nanotubes in male mice cause reversible testis damage without affecting fertility. Nat Nanotechnol. 2010; 5:683-689.
21. Chandra AK, Chatterjee A, Ghosh R, Sarkar M. Effect of curcumin on chromium-induced oxidative damage in male reproductive system. Environmental Toxicology and Pharmacology. 2007; 24:160-166.
22. Pothuraju R, Gautam K. Effect of Silver Nanoparticles on Functionalities of Buffalo (*Bubalus bubalis*) Spermatozoa. Advanced Science, Engineering and Medicine. 2013; 5:91-95.
23. Taylor U, Barchanski A, Petersen S, Kues WA, Baulain U, Gamrad L. Gold nanoparticles interfere with sperm functionality by membrane adsorption without penetration. Nanotoxicology. 2014; 8:118-27.
24. Aporvari AMH, Mamoei M, Tabatabaei Vakili S, Zareei M, Dadashpour Davachi N. The Effect of Oral Administration of Zinc Oxide Nanoparticles on Quantitative and Qualitative Properties of 1 Arabic Ram Sperm and Some Antioxidant Parameters of Seminal Plasma in the Non-Breeding Season. Archives of Razi Institute. 2018; 73(2):121-129.
25. Falchi L, Galleri G, Dore GM, Zedda MT, Pau S, Bogliolo L. Effect of exposure to CeO2 nanoparticles on ram spermatozoa during storage at 4 degrees C for 96 hours. Reprod Biol Endocrinol. 2016; 16:19.
26. Makhluf SBD, Arnon R, Patra CR, Mukhopadhyay D, Gedanken A, Mukherjee P. Labeling of sperm cells via the spontaneous penetration of Eu3þ ions as nanoparticles complexed with PVA or PVP. J Phys Chem. 2008; 112:12801-7.
27. Makhluf SBD, Qasem R, Rubinstein S, Gedanken A, Breibart H. Loading magnetic nanoparticles into sperm cells does not affect their functionality. Langmuir. 2006; 22(23):9480-9482.
28. Skovmand A, Lauvas AJ, Christensen P, Vogel U, Hougaard KS, Goericke-Pesch S. Pulmonary exposure to carbonaceous nanomaterials and sperm quality. Particle and Fibre Toxicology. 2018; 15:10.
29. Torabi F, Shafaroudi MM, Rezaei N. Combined protective effect of zinc oxide nanoparticles and melatonin on cyclophosphamide induced toxicity in testicular histology and sperm parameters in adult Wistar rats. Int J Reprod Biomed. 2017; 15(7):403-412.
30. Taylor U, Petersen S, Barchanski A, Mittag A, Barcikowski S, Rath D. Influence of gold nanoparticles on vitality parameters of bovine spermatozoa. Reprod Domest Anim. 2010; 45:60-60.
31. Barkalina N, Jones C, Kashir J, Coote S, Huang X, Morrison R *et al*. Effects of mesoporous silica nanoparticles upon the function of mammalian sperm *in vitro*. Nanomedicine. 2014; 10(4):859-870.
32. Tiedemann D, Taylor U, Rehbock C, Jakobi J, Klein S, Kues WA *et al*. Reprotoxicity of gold, silver, and gold-silver alloy nanoparticles on mammalian gametes. Analyst. 2014; 139(5):931-42.
33. Feugang JM, Youngblood RC, Greene JM, Fahad AS, Monroe WA, Willard ST *et al*. Application of quantum dot nanoparticles for potential non-invasive bio-imaging of mammalian spermatozoa. Journal of Nanobiotechnology. 2012; 10:45.
34. Swain DK, Tarai A, Mohapatra APK, Kundu AK. Evaluation of longevity of canine epididymal spermatozoa stored at 4°C. Indian Journal of Veterinary Research. 2012; 20(1):16-20.
35. Tarai A, Swain DK, Mohapatra APK, Kundu AK. *In vitro* study of longevity of canine epididymal sperm at 25°C. Indian Veterinary Journal. 2010; 87:1198-1200.
36. Taylor U, Petersen S, Barchanski A, Mittag A, Barcikowski S, Rath D. Influence of gold nanoparticles on vitality parameters of bovine spermatozoa. Reprod Domest Anim. 2012; 45:60-60.
37. Nazari M, Talebi AR, Sharifabad MH, Abbasi A,

Khoradmehr A, Danafar AH. Acute and chronic effects of gold nanoparticles on sperm parameters and chromatin structure in Mice. Int J Reprod Biomed. 2016; 14(10):637-642.
38. Abbasalipourkabir R, Moradi H, Zarei S, Asadi S, Salehzadeh A, Ghafourikhosroshahi A *et al*. Toxicity of zinc oxide nanoparticles on adult male Wistar rats. Food and Chemical Toxicology. 2015; 84:154-60.
39. Vasquez J, Florentini EA, Camargo LA, Gonzales J, Valdivia M. Hypoosmotic swelling test in epididymal ram (*Ovis aries*) spermatozoa. Livestock Science. 2013; 157(2-3):618-622.
40. Mathias FT, Romano RM, Kizys MM, Kasamatsu T, Giannocco G, Chiamolera MI *et al*. Daily exposure to silver nanoparticles during prepubertal development decreases adult sperm and reproductive parameters. Nanotoxicology. 2015; 9(1):64-70.
41. Panatarotto D, Prtidos CD, Hoebeke J, Brown F, Kramer E, Briand JP *et al*. Immunization with peptide-functionalized carbon nanotubes enhances virus-specific neutralizing antibody responses. Chemistry & Biology. 2003; 10:961-966.
42. Omu AE, Dashti H, AI Othman S. Treatment of asthenozoospermia with ZnSO4: andrological immunological and obstetric outcome. Eur. J. Obst. Gynae. Reprod. BioI. 1998; 79:179-184.