Chapter-3

Toxicity study of biogenic gold nanoparticles (AuNPI3Cs)

3.1 Introduction

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Abstract

Environmental remediation, gene therapy, diagnostics, sensory purposes, detection of actuate materials in live cells and various types of field have been developed by nanotechnology. With the appearance of these nanoparticles in the environment, their toxicity assessment is an immediate concern.

This study was initiated to enhance our insight on the health and environmental impact of biogenic gold nanoparticles (AuNPI3Cs). The present study was aimed to assess the *in vitro* toxicity of AuNPI3Cs using brine shrimp, zebra fish and human lymphocytes and also to examine *in vivo* sub-acute toxicity in mice. In brine shrimp, LC_{50} of AuNPI3Cs was 2500 μgml^{-1}. At 3000 μgml^{-1} of AuNPI3Cs, slight edema was observed on abdominal area of zebra fish embryos.

ROS generation was not found in AuNPI3Cs treated human lymphocytes which confirmed that AuNPI3Cs did not induce oxidative stress in normal lymphocytes. AuNPI3Cs exhibited no genotoxic effect on brine shrimp and human lymphocytes. The sub-acute toxicity of AuNPI3Cs was evaluated at 50, 100, 250, 500, 2000 and 4000 µg/kg body weight in Swiss albino mice for 28 days. In AuNPI3Cs treated mice, body weight, haematological, hepatic and renal biomarkers were not altered up to 2000 µg/kg body weight compared to control animals.

Histopathology examinations of liver, kidney, spleen, and testis did not reveal any morphological changes. From the above findings it can be concluded that AuNPI3Cs treatment did not show any toxic effect both *in vitro* and *in vivo* model.

3.1 Introduction

The toxicology field is a branch of science that deals with poisons and their effects. Toxicity screening is very crucial for the development of new drugs. The US Food and Drug Administration (FDA) reports that it is significant to verify new molecules for pharmacological activity and toxicity screening in animals. The toxic effects of drugs, pharmaceuticals have attained very much significance in the 21 century.

3.1.1 Types of toxicity test

Acute toxicity study is evaluated to determine the effect of a single dose on animal species. To study the sub-chronic toxicity of a compound rodents and non-rodents are usually used and for 90 days the test substance is administered orally. Then change in body weight, biochemical and cardiovascular parameters are generally examined.

A chronic toxicity study reports about the long-term effect of a test substance in animals, and it may be considered to the human safety of the test substance.

3.1.2 Modes of toxicity assessment

Zebra fish

Zebra fish has been used mostly in developmental biology, but their value in toxicology as well as in drug discovery has been recognised. Zebra fish (*Danio rerio*) has been considered as a good model in biological fields. There are numerous advantages for the use of zebra fish as a toxicological model (Spitsbergen and Kent, 2003).

***Artemia salina* (brine shrimp)**

Artemia is a non-selective organism which is available in highly saline waters. Due to their universal distribution and cost effective culture they are considered as a good model

organism for toxicity testing. Artemia are considered to be one of the impervious models for testing the chemicals. Few studies are reported for nanomaterial toxicity using Artemia sp. (Ates et al., 2013, Rajabi et al., 2015).

3.1.3 Nanotoxicology

Nanotoxicology is a branch of bio-nanoscience, which deals with the application of the toxicity of nanomaterials. To determine whether and to what extent nano materials may pose a threat to the environment and to human health nanotoxicological studies are used (Buzea et al., 2007). Nanoparticles play a significant role in toxicity, which is important for toxicologists.

Some of the particle features and oxidative stress play important roles in nanotoxicity. By gaining control over toxic materials, we can increase the use of nanoparticles and thus agreeing them to be used for the treatment of diseases.

In this chapter, the toxicity study of AuNPI3Cs was evaluated. Acute toxicity was assessed on brine shrimp, zebrafish, human lymphocyte and sub-acute toxicity study was performed in mice model evaluating haematological parameters, serum biochemistry and histopathological changes.

3.2 Materials and methods

3.2.1 Chemicals

ovine serum albumin (BSA), sodium carbonate ($NaHCO_3$), copper sulphate ($CuSO_4$), sodium potassium tartarate, sodium hydroxide (NaOH), folin-Ciocalteau solution, DL-aspartate, α ketoglutarate, DL-alanine, glacial acetic acid, dinitrophenyl hydrazine (DNPH), diacetylmonoxime, sodium tungstate, urea, picric acid, p-nitrophenol phosphate (PNPP),cholesterol, ferric chloride ($FeCl_3$), 5-Dithiobis-(2-nitrobenzoic acid) (DTNB), Tris

buffer, titron X- 100, Tris–HCl, phenol, iso-amyl alcohol, chloroform, haematoxylin, eosin and other chemicals were procured from Merck India, Ltd., Mumbai, India for experimentation.

3.2.2 *In-vitro* acute toxicity study

3.2.2.1 *In-vitro* toxicity study on zebrafish embryos

Zebrafish **(***Danio rerio***)** is a vertebrate model for toxicology, pharmacology, developmental biology and genetics research (Parng et al., 2002). Zebrafish is a tropical fresh water fish which belongs to the cyprinidae family. Zebrafish favours basically warm water, but they increase in many environments.

Collection of zebra fish embryo

For toxicity studies, 10 healthy cultured zebrafish embryos were transferred to the wells of a 96-well plate along with 250µl of brine water (60 mg of sea salt/liter of millipore water). Different concentrations of nanoparticles (5, 10, 25, 50, 100, 250, 500, 1500, 3000 µg ml^{-1}) were added to the wells and incubated for 48 h at 28±1˚C. Tests were performed in triplicate manner.

Procedure of toxicity study on zebrafish embryo

AuNPI3Cs in different concentration were applied in 96-well plates through pipette. After 24 and 48 hpf, the plates were observed under inverted microscope (Carlesson et al.,2011). Mortality of the embryos was noted after 24, 48 h. The mortality rate is expressed as the total number of dead embryos after 24, 48 h. The emergence of pericardial edema and deformities were recorded.

3.2.2.2 Brine shrimp lethality test

Artemia salina, a type of salt-water shrimp invertebrate **(**Mirzaei and Mirzaei, 2013**)** is used for brine shrimp [*Artemia salina* (*A.salina*)] lethality test (BSLT). Cytotoxicity (McLaughlin, 1991) and bioactivity of a material is simply determined by brine shrimp lethality assay (Sorgeloos, 1978).

Collection and hatching of brine shrimp

Artemia salina eggs were collected from West Bengal Comprehensive Development. Eggs were placed in a container containing artificial sea water which was prepared by dissolving 35 g of sodium chloride in 1 L of distilled water. After 36–48 h incubation at room temperature (28–30°C) under conditions of strong aeration and continuous illuminations (Sangian et al., 2013), the larvae (nauplii) was hatched within 48 h.

Procedure of brine shrimp lethality test

The evaluation of cytotoxicity of AuNPI3Cs in *A. salina* was performed according to the method of Sangian et al., 2013. The assay was carried out on larvae of brine shrimp. Then, fresh suspensions with different concentrations of AuNPI3Cs were made by means of serial dilution of the stock suspensions of AuNPI3Cs in artificial sea water. Then 200 μL of different concentrations of AuNPI3Cs were added to each well of the 96-well plates. After that, 10 nauplii per well were added in the 96-well plates and incubated at room temperature for 24 h. The numbers of surviving nauplii in each well were counted under a inverted microscope after 24 h. The experiments were conducted in triplicate for each concentration. The negative control wells contained 10 nauplii and artificial sea water only.

Percentage of mortality i.e. death rate of *A. salina* nauplii = (No. of dead nauplii in test group - No. of dead nauplii in control group) /No. of living nauplii in control group) x 100

3.2.2.3 Toxicity study on human lymphocytes

Collection, separation and culture of human lymphocytes

Human blood samples were collected from healthy individual in 5 ml heparin-coated vacutainers (Hudson and Hay, 1989). Then 1 ml of Histopaque 1077 was mixed with same amount of collected blood. The mixture was centrifuged for 30 min at room temperature at 2000 rpm and the upper buffy monolayer having lymphocytes were separated. It was then transferred to a clean centrifuge tube. After washing thrice in phosphate buffer solution (PBS) the human lymphocytes (HLCs) were suspended in RPMI-1640 media having 10% FBS. It was then incubated in a CO_2 incubator at 37°C for 24 h under 5% CO_2 and 95% humidified atmosphere.

Oxidative stress study on human lymphocytes

MDA Estimation

Lymphocyte MDA was determined by the method of Ohkawa et al., 1979. Firstly, lymphocytes were treated with AuNPI3Cs for 24h. After 24h, the cell pellet was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of acetate buffer (20% pH 3.5), and 1.5 ml of aqueous solution of thiobarbituric acid. Red pigment was produced after heating at 95°C for 60 min and it was extracted with 5 ml of *n*-butanol-pyridine mixture (15:1) and centrifuged at 5000 rpm for 10 min at room temperature. The optical density of supernatant was measured at 535 nm.

Estimation of reduced glutathione (GSH)

After 24h treatment of AuNPI3Cs, lymphocytes were washed with PBS and 100 µl of 4gm % sulfosalicylic acid was added with 200 µl of each lymphocyte sample. Then it was centrifuged for 10 min at 2000 rpm and 2 ml of 0.6 mM DTNB was mixed to 200 µl of

supernatant. The reaction of GSH and DTNB yields a yellow-coloured complex which was measured at 412-420 nm (Griffith 1981).

Intracellular ROS measurement

After treatment of AuNPI3Cs for 24 h, cells were washed with phosphate buffer and incubated with 1 μg ml^{-1} H_2DCFDA for 30 min at 37ºC and washed again three times with culture media. At 485 nm excitation and 520 nm emission, DCF fluorescence intensity was measured in Hitachi F-7000 fluorescence spectrophotometer and under fluorescence microscope (LEICA DFC295, Germany) the cells were also detected. The experiment was done three times (Roy et al., 2008).

DNA fragmentation study

After 24 h treatment, lymphocytes were washed with phosphate buffer saline (pH 7.4) and 0.7% agarose was mixed with the cell pellet. After that the sample was transferred onto a 1% agarose smeared glass slide and slides were poured into lysis buffer. It was then electrophorized for 20 min and in neutralization buffer the slides were then neutralized. It was stained with ethidium bromide and observed under fluorescence microscope (LEICA DFC295, Germany). Tail lengths were determined as the percentage of DNA in each tail and % DNA (tail) stated as: Tail area × Tail area intensity× 100/ [(Tail area × Tail area intensity) + (Head area × Head area intensity)]. The experiment was performed three times (Alcˆantara et al., 2011).

Analysis of cell cycle by flow cytometry

At first, 24 h AuNPI3Cs-treated lymphocytes were centrifuged for 5 min at 1000-1200 rpm. The cells were fixed in 70% chilled ethanol after PBS washing and the samples were centrifuged for 5 min at 2000 rpm. After washing twice in ice-cold PBS the cells were incubated with RNAse (10 mg ml^{-1}) solution at 37ºC for 1 h. The cells were stained with

propidium iodide (1mg ml^{-1}) and after PBS washing diluted with 500 µl PBS (Evans et al., 2000). Using flow cytometer (BD FACSVerse) and Cell Quest software, the cell cycle was analyzed.

3.2.2.4 Sub-acute toxicity study in mice model

Animal maintenance

For toxicity test, healthy male Swiss albino mice (18–25 g) were maintained under standard laboratory condition [temperature (25 ± 2°C) and humidity (60 ± 5%) with 12 h light /dark cycle] and a standard pellet diet and drinking water was supplied. The study was ratified by the Institutional Animal Ethical Committee under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (approval No. IEC/7-14/C14-16).

Treatment schedule

The sub-acute toxicity study was carried out according to OECD guideline 407. Adult healthy male Swiss albino mice were divided into seven groups of 5 animals each and were placed under standard laboratory conditions. AuNPI3Cs were administered intraperitoneally (Coria-Avila et al.,2007) at the dose levels of 50, 100, 250, 500, 2000 and 4000 µg/kg body weight to the group II to VII animals respectively by sterile syringe daily for 28 days and Group I was considered as saline control (Sunday et al.,2013 and Hosseinzadeh et al.,2013).

Group I : Saline control

Group II A & B: I3C (50 mg/kg body weight) & AuNPI3Cs (50 µg/kg body weight)

Group III A & B: I3C (100 mg/kg body weight) & AuNPI3Cs (100 µg/kg body weight)

Group IV A & B: I3C (250 mg/kg body weight) & AuNPI3Cs (250 µg/kg body weight)

Group V A & B: I3C (500 mg/kg body weight) & AuNPI3Cs (500 µg/kg body weight)

Group VI A & B: I3C (2000 mg/kg body weight) & AuNPI3Cs (2000 µg/kg body weight)

Group VII A & B: I3C (4000 mg/kg body weight) & AuNPI3Cs (4000 µg/kg body weight)

Body and organ weight measurement

Body weight of all the animals was recorded and then they were sacrificed on 29th day. Different organs i.e. lungs, heart, liver, spleen, and kidneys were dissected out carefully and weighed in grams.

Blood sample collection

On 29th day, blood samples were collected using a 5 ml sterile syringe from all animals. For haematological parameters, 2 ml blood sample was taken into EDTA containing sterile container. For serum analysis, another portion of blood samples were collected into anticoagulant free sterile containers. Then the samples were centrifuged at 2000 rpm for 10 min. The supernatants were obtained from sterile containers and stored at -20^{0} C for further biochemical analysis.

Red blood cell (RBC) count

Total RBC were counted using Neubaur haemocytometer. Blood was diluted 1:200 with RBC dilution fluid and charged in the haemocytometer chamber to count (Wintrobe, 1967).

White blood cell (WBC) count

Blood was diluted in 1: 20 ratio with WBC dilution fluid and charged in haemocytometer chamber. Large squares of the four corner of haemocytometer chamber were counted under the microscope (Wintrobe, 1967).

Determination of haemoglobin percentage

The haemoglobin percentage was estimated by cyanmethaemoglobin method. At first 20 µl of blood was added into a test tube containing 5 ml of Drabkin's solution. The optical density of sample was measured at 540 nm (Dacie and Lewis, 1975).

Estimation of urea

Urea was determined by the modified method of Natelson (Natelson et al., 1951). At first, 0.1 ml of serum was taken and 3.3 ml of water, 0.3 ml each of 10% sodium tungstate and 0.67 N sulphuric acid was added to it. The suspensions were centrifuged at 2000 rpm. Then 1.0 ml of water, 2.6 ml of 0.67 N sulphuric acid-phosphoric acid and 0.4 ml of diacetylmonoxime were added and mixed to the supernatant. Standard urea (20 to 50 µg/ml) was also prepared similarly. All the test tubes were heated for 30 min in a boiling water bath and cooled. The color intensity was estimated at 480 nm in spectrophotometer (UV-Shimadzu-245, Japan). The results were expressed as mg of urea/dl of blood.

Estimation of creatinine

For the measurement of creatinine (Brod and Sirota, 1948) 8.0 ml of water, 0.5 ml of 2/3 N sulphuric acid and 0.5 ml of 40% sodium tungstate was added with 1 ml of serum. Then to 5.0 ml clear filtrate, 1.5 ml of saturated picric acid and 1.5 ml of 0.75N sodium hydroxide were mixed well. The color intensity was noted at 530 nm and the results were stated as mg% of creatinine.

Measurement of serum alkaline phosphatase (ALP)

In a centrifuge tube, 0.25ml of serum was mixed with 1 ml of buffer containing 1 mM of p-nitrophenol phosphate in 1M Tris buffer, pH 8.0 and was incubated for 30 min at 37ºC in a water bath. The serum alkaline phosphatase activity was measured at 420 nm (Malamy and Horecker, 1966).

Measurement of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT)

At first, 1.0 ml of SGOT buffer substrate (200 mM/L of DL-aspartate and 2 mM/L of α-ketoglutarate, pH 7.4) was added to 0.1 ml of serum and incubated for 1 h at 37°C followed by addition of 1ml of dinitrophenyl hydrazine (DNPH).The mixture was kept for 20 min at room temperature. Then, 10 ml of 0.4N sodium hydroxide was added to that mixture after 20 min and allowed to stand for another 10 min. The developed color was measured at 505-540 nm spectrophotometrically (UV-Shimadzu-245, Japan) against blank.

For the estimation of SGPT, 0.1 ml of serum was taken and 1.0 ml of buffer substrate (200 mM/l of DL-alanine and 2 mM/L of α- ketoglutarate pH=7.4) was added to it and incubated at 37°C for 1 h. Then, 1.0 ml of dinitrophenyl hydrazine was added to that mixture and kept for 20 min at room temperature. After that 10 ml of 0.4N sodium hydroxide was added and the formation of colour was read at 505-540 nm in spectrophotometer (UV-Shimadzu-245, Japan) against blank. The enzyme activity was expressed as IU/lit (Goel, 1988).

Blood glucose estimation

Firstly, 24 g of anhydrous sodium carbonate and 12 g of sodium potassium tartrate were dissolved in 250 ml of distilled water. After that, 4 gm of 10% (w/v) copper sulphate solution was added to it followed by the mixing of 16 g of sodium bicarbonate. Then 180 g of sodium sulphate was dissolved in 500 ml of distilled water and boiled. After cooling, the two solutions were mixed thoroughly and the volume was made up to 1000 ml. Arsenomolybdate reagent was made up by dissolving 25 g ammonium heptamolybdate in 450 ml of distilled water followed by addition of 21 ml sulphuric acid to it. Disodium hydrogen arsenate was added to it and mixed well. Then the solution was incubated at 37°C for 24 h. One ml of aliquot was pipetted out and 1.0 ml of Somogyi's copper reagent was added to it. The mixture

was then placed in a water bath and heated for 20 min. After cooling, 1.0 ml of Nelson's arsenomolybdate reagent was added. The color intensity was measured spectrophotometrically at 540 nm (Nelson, 1944 and Somogyi, 1952).

Estimation of serum cholesterol

Firstly, 0.1 ml of serum was taken and mixed with 6 ml of glacial acetic acid. Then 4 ml of color reagent (1 ml of 10% $FeCl_3$, 6 H_2O, 15 ml of conc. H_2SO_4) was added to it and allowed to stand for 20 min. The reading was taken at 570nm. The cholesterol is calculated by plotting the standard curve (Zlatkis et al., 1953).

Histopathological study

For histopathological examinations, organs such as liver, kidney were collected from sacrificed animals of all the groups. The collected organs were dipped into 10% neutral buffered formalin. Then the tissues were dehydrated in graded alcohols and embedded in paraffin. Tissues were cut in five micron thickness and the sections were stained with haematoxylin and eosin (H and E) (Standish et al., 2006).

3.2.2.5 Statistical analysis

All the experiments were performed thrice and the results were stated as Mean ± SEM. By using one-way ANOVA test, comparison were calculated between the means of the control and treated groups (using a statistical package, Origin 6.1, Northampton, MA), $p<0.05$ as a limit of significance.

3.3 Results

3.3.1 Acute toxicity study of AuNPI3Cs

3.3.1.1 Effect of AuNPI3Cs on toxicity study of zebrafish embryos

In zebrafish toxicity study, after treatment of AuNPI3Cs no significant morphological alteration was observed at tail, head, and eye. Minor oedema was detected on abdominal area and thoracic cavity of zebrafish embryos at 3000µg ml^{-1} of AuNPI3Cs (Table 3.2).

Table: 3.1. Effect of AuNPI3Cs on morphological toxicity of Zebrafish embryo

Observation	Control	AuNPI3Cs (µg/ml)						
		25	50	100	250	500	1000	2000
24 hpf								
Number of embryos	10	10	10	10	10	10	10	10
Tail Development	0	0	0	0	0	0	0	0
Eye Development	0	0	0	0	0	0	0	0
Head Development	0	0	0	0	0	0	0	0
No. of affected	0	0	0	0	0	0	0	1
Edema formation	0	0	0	0	0	0	0	1

3.3.1.2 Effect of AuNPI3Cs on Brine shrimp lethality bioassay

The LC_{50} value of AuNPI3Cs against brine shrimp lethality test was 2500µg/ml (Table 3.2) after 24 h treatment.

Table 3.2 shows the percentage of mortality (24 h) and determination of LC_{50} dose of AuNPI3Cs against brine shrimp

Sample	Concentration(µg/ml)	% of mortality	LC_{50}(µg/ml)
	5	0	
	10	0	
	25	0	
	50	0	
	100	0	
	250	3.33%	
	500	16.66%	
	1500	33.33%	
	3000	60%	

3.3.1.3 Effect of AuNPI3Cs on brine shrimp morphology

It was found that there was no significant morphological changes occurred in AuNPI3Cs treated groups of brine shrimps compared to control group.

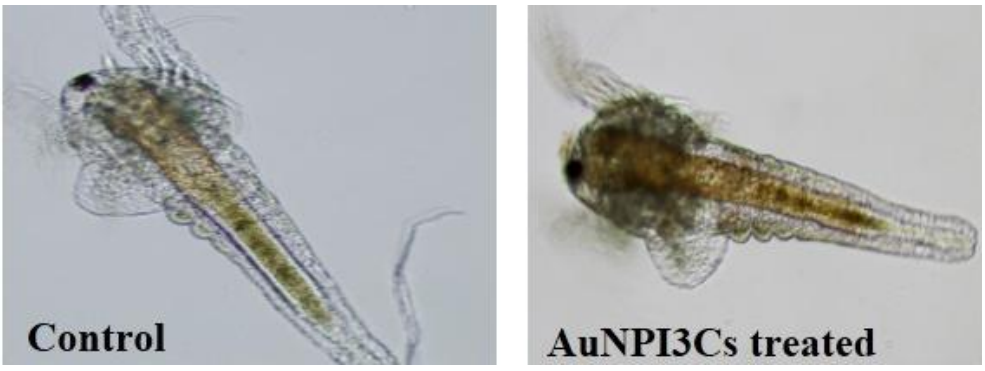


Figure 3.1.Morphological changes of brine shrimp after treatment of AuNPI3Cs for 24 h.

3.3.1.4 Effect of AuNPI3Cs on comet assay of brine shrimp

AuNPI3Cs treated brine shrimp exhibited no significant increase in percentage of DNA tail intensity compared to the control group.

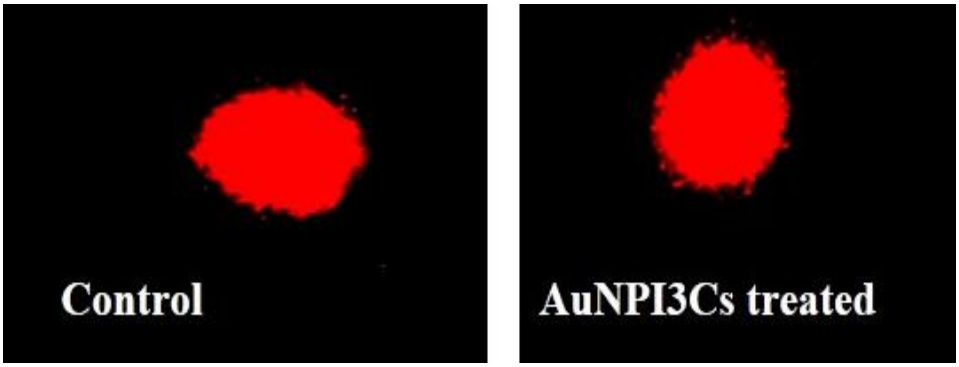


Figure 3.2. Comet assay of Brine shrimp after AuNPI3Cs treatment

3.3.2 Toxicity study of AuNPI3Cs on human lymphocytes

3.3.2.1 Effect of AuNPI3Cs on MDA level of human lymphocytes

It was found that MDA levels were not altered significantly in AuNPI3Cs treated groups compared to lymphocyte control group.

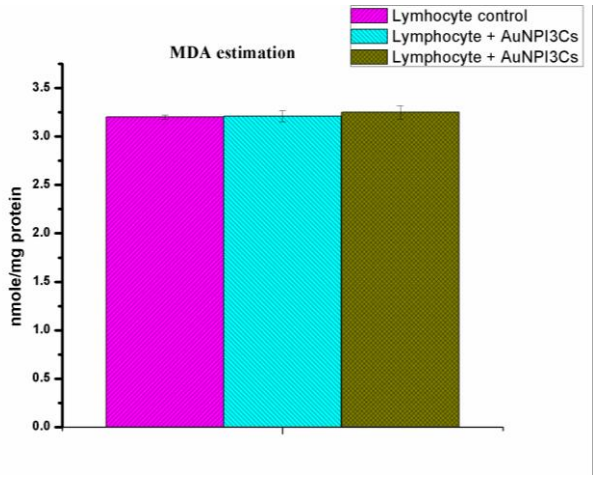


Figure 3.3. The effect of AuNPI3Cs on lymphocyte MDA level. The levels of MDA were expressed as nmole mg^{-1} protein. Values are expressed as Mean ± SEM.

3.3.2.2 Effect of AuNPI3Cs on GSH level of human lymphocytes

It was noted that GSH level was not altered significantly in AuNPI3Cs treated group compared to control (Figure 3.4).

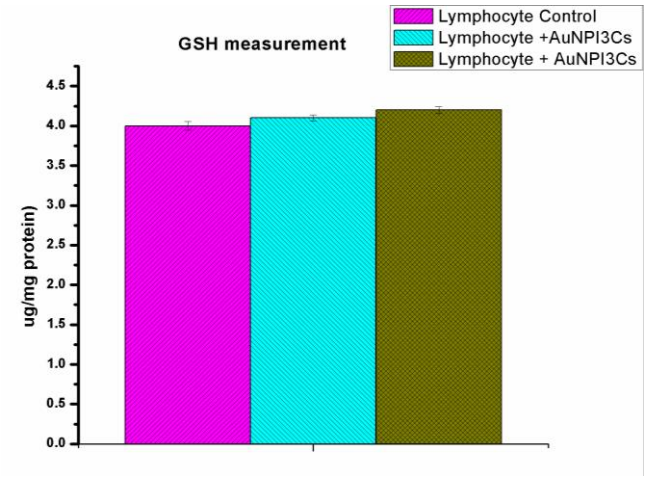


Figure 3.4. The effect of AuNPI3Cs on lymphocyte intracellular reduced glutathione level (GSH). GSH levels were expressed as μg mg^{-1} protein. Values are expressed as Mean ± SEM.

3.3.2.3 Effect of AuNPI3Cs on ROS generation of human lymphocytes

It was found that fluorescence intensity was not increased significantly in AuNPI3Cs treated group compared to control (Figure 3.5).

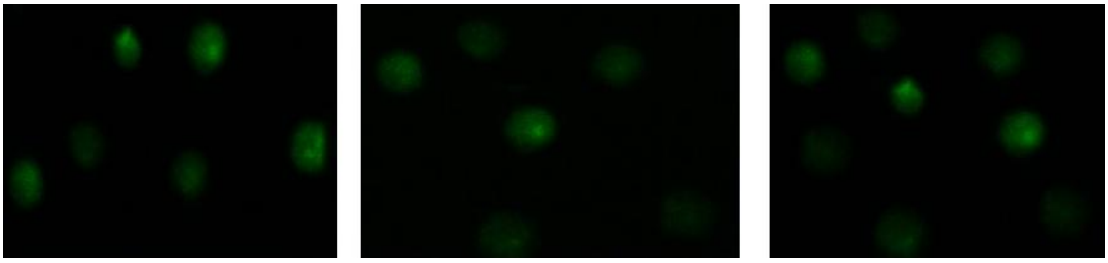


Figure 3.5. Intracellular reactive oxygen species (ROS) generation of AuNPI3Cs treated human lymphocytes by H_2DCFDA staining.

3.3.2.4 DNA fragmentation study of human lymphocytes

Genotoxic effect of AuNPI3Cs on human lymphocytes was determined by alkaline comet assay. AuNPI3Cs treated lymphocytes were examined under fluorescence microscope and it was found that there was no significant increase of tail DNA intensity compared to control (Figure 3.6).

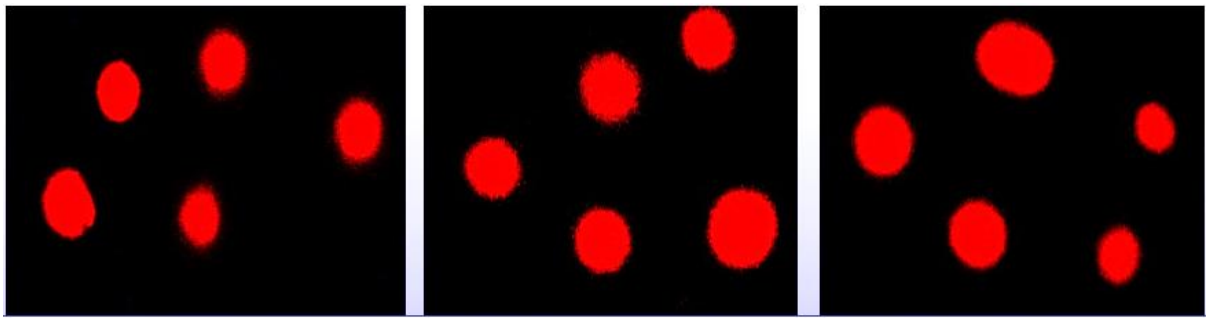


Figure 3.6. Determination of the genotoxic effects of AuNPI3Cs in human lymphocytes by alkaline comet assay.

3.3.2.5 Cell cycle analysis of lymphocyte by flow cytometry

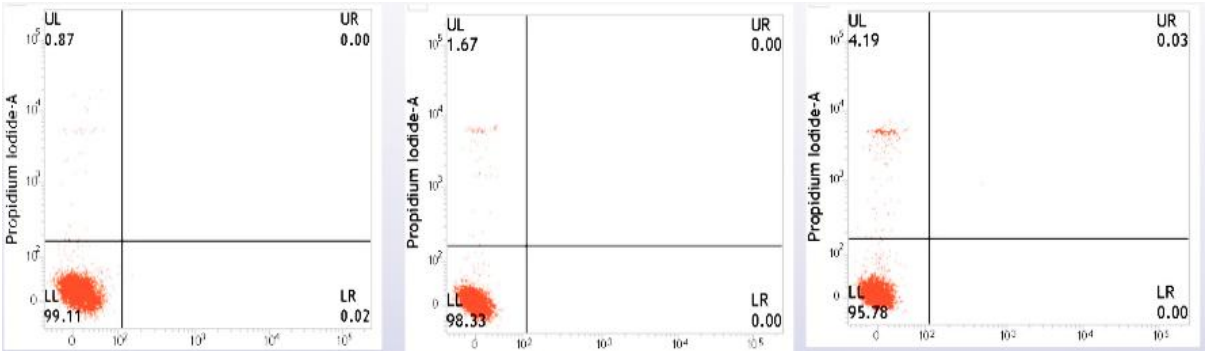


Figure 3.7. Dot plots of cell cycle distribution of AuNPI3Cs treated human lymphocytes.

3.3.4. Sub-acute toxicity study in *in vivo*

3.3.4.1 Effect of AuNPI3Cs on changes in body weight and organ weight

It was found that there was no significant difference in body weight and relative organs weight in AuNPI3Cs treated group up to dose level of 4000 µg/kg bwt (Table 3.3). It was revealed that AuNPI3Cs has no harmful effects on vital organs such as liver, kidney, heart and spleen.

Table 3.3: Effect of AuNPI3Cs administration on body weight and different organ weight of mice

	Saline control	50 µg/kg bwt	100µg/kg bwt	250µg/kg bwt	500µg/kg bwt	2000µg/kg bwt	4000µg/kg bwt
Initial Body weight (g)	**27.66±0.33**	**27±0.57**	**27.7±0.33**	**27.88±0.57**	**28±0.57**	**28±0.57**	**28±0.57**
Final Body weight (g)	**31±0.57**	**31±0.57**	**31±0.57**	**31±0.57**	**32±0.57**	**32±0.57**	**35±0.57**
Heart weight (g)	**0.22±.005**	**0.22±.005**	**0.22±.005**	**0.21±.005**	**0.21±.003**	**0.2±.003**	**0.19±.005**
Spleen weight (g)	**0.24±.008**	**0.24±.008**	**0.26±.005**	**0.27±.003**	**0.29±.005**	**0.29±.008**	**0.32±.011***
Liver weight (g)	**0.33±.008**	**0.33±.008**	**0.34±.008**	**0.36±.005**	**0.36±.008**	**0.41±.008**	**0.41±.011**
Kidney weight (g)	**0.38±.003**	**0.36±.005**	**0.36±.005**	**0.35±.005**	**0.35±.005**	**0.32±.003**	**0.3±.005**

Results are expressed as Mean ± SEM.

3.3.4.2 Effect of AuNPI3Cs on haematological parameters

The results of the haematological parameters are documented in Table 3.4. It was found that haematological parameters i.e. haemoglobin percentage, red blood cell count, white blood

cell count were not changed in AuNPI3Cs treated groups up to 4000µg/kg bwt compared to saline control group.

Table 3.4: Haematological parameters after AuNPI3Cs administration

Hematological Parameters	Saline control	50 µg/kg bwt	100µg/kg bwt	250µg/kg bwt	500µg/kg bwt	2000µg/kg bwt	4000µg/kg bwt
Hb Percentage	**15±0.577**	**15±0.55**	**14.8±0.25**	**14.5±0.4**	**14.5±0.54**	**14±0.55**	**13.5±0.54**
Total RBC Count(×106mm3)	**6±0.145**	**6±0.012**	**5.8±0.104**	**5.8±0.05**	**5.5±0.057**	**5.1±0.04**	**5.1±0.02**
Total WBC Count(/µl)	**5100±58**	**5100±50**	**5200±62**	**5300±60**	**5300±78**	**5400±65**	**5560±70**

Results are expressed as Mean ± SEM.

3.3.4.3. Effect of AuNPI3Cs on hepatic and renal biochemical parameters

The results of various biochemical parameters of AuNPI3Cs treated mice and saline control group are represented from Figure 3.8 to 3.15. Intraperitoneal administration of AuNPI3Cs at the dose of 50, 100, 250, 500, 2000, 4000µg/kg body weight did not cause any significant changes in biochemical parameters such as urea, creatinine, SGOT and SGPT levels, blood glucose, and serum cholesterol compared to saline control group.

3.3.4.3.1 Changes in serum urea level

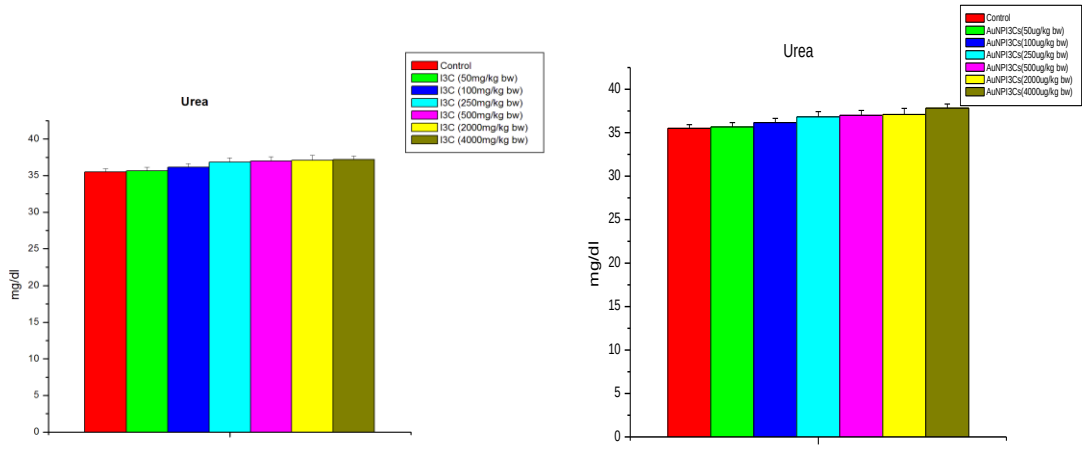


Figure 3.8. The effect of I3C and AuNPI3Cs on serum urea level of Swiss albino mice. Values are expressed as Mean ± SEM; n=6.

3.3.4.3.2 Changes in serum creatinine level

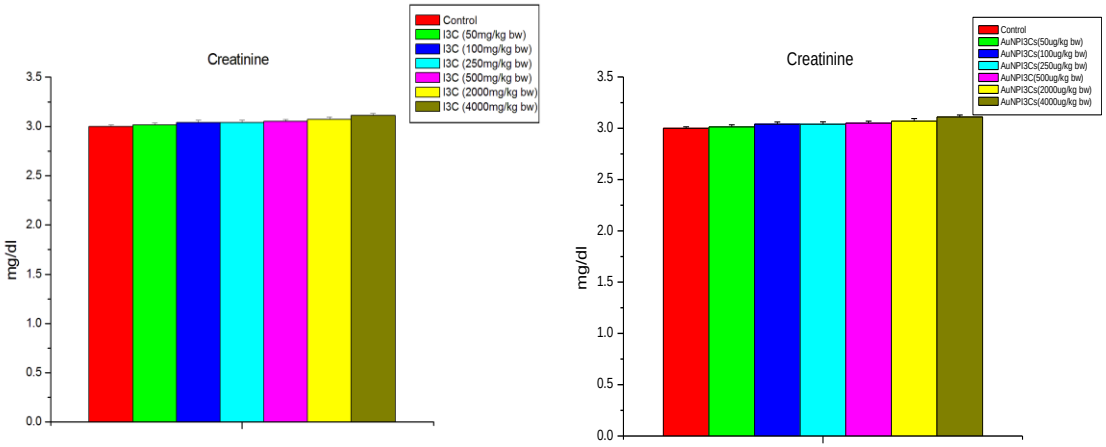


Figure 3.9 shows the effect of I3C and AuNPI3Cs on serum creatinine level of Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one way ANOVA.

3.3.4.3.3 Changes in serum ALP level

Figure 3.10 shows the effect of I3C and AuNPI3Cs on serum ALP level of Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one way ANOVA.

3.3.4.3.4 Changes in SGOT level

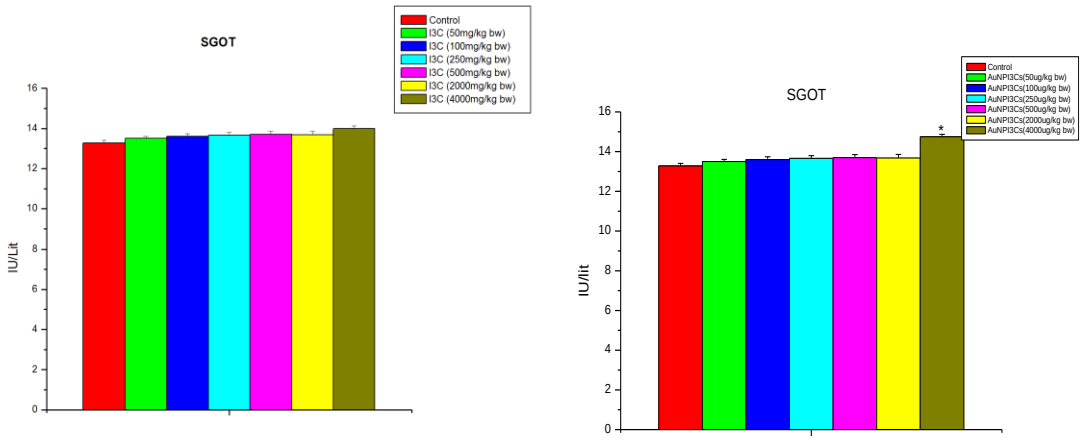


Figure 3.11 shows the effect of I3C and AuNPI3Cs on serum glutamic oxaloacetic transaminase (SGOT) of Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one way ANOVA. '*'indicate significant difference ($p<0.05$) compared to control.

3.3.4.3.5 Changes in SGPT level

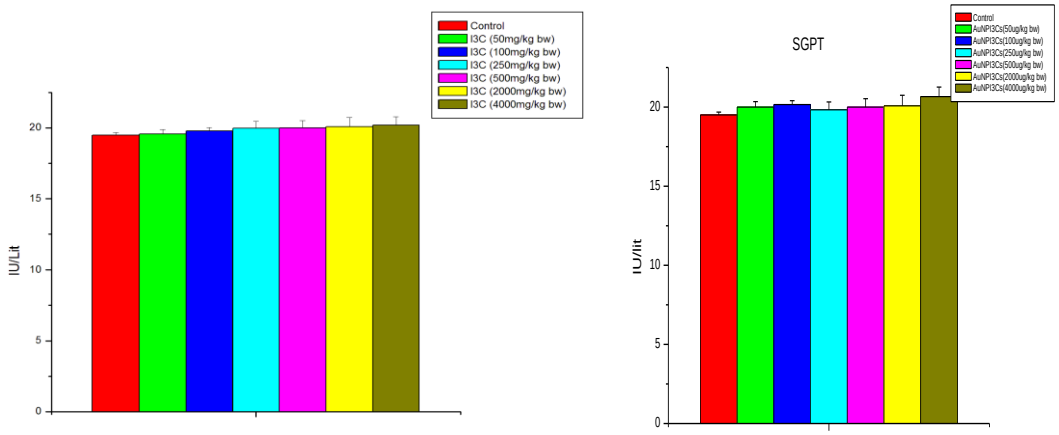


Figure 3.12. The effect of I3C and AuNPI3Cs on serum glutamic pyruvic transaminase (SGPT) of Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one way ANOVA.

3.3.4.3.6 Changes in blood glucose level

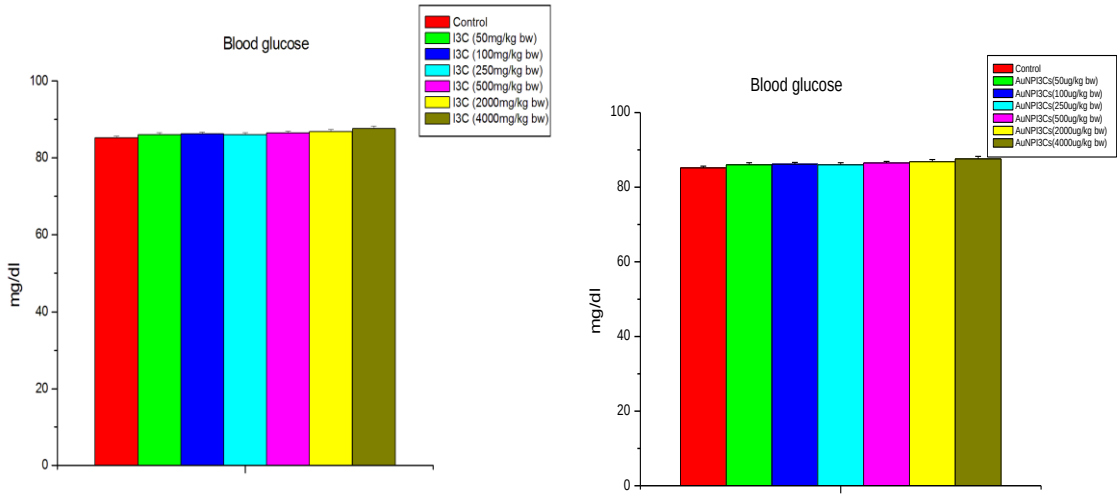


Figure 3.13 shows the effect of I3C and AuNPI3Cs on blood glucose level of Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one way ANOVA.

3.3.4.3.7 Changes in serum cholesterol level

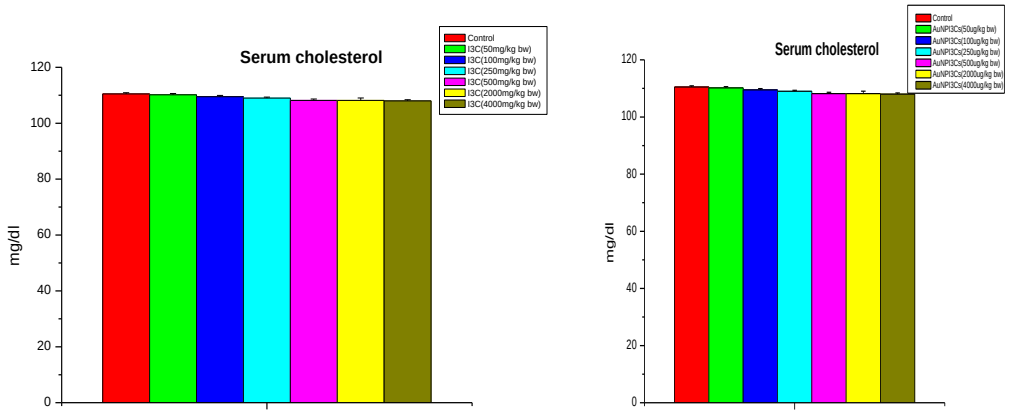
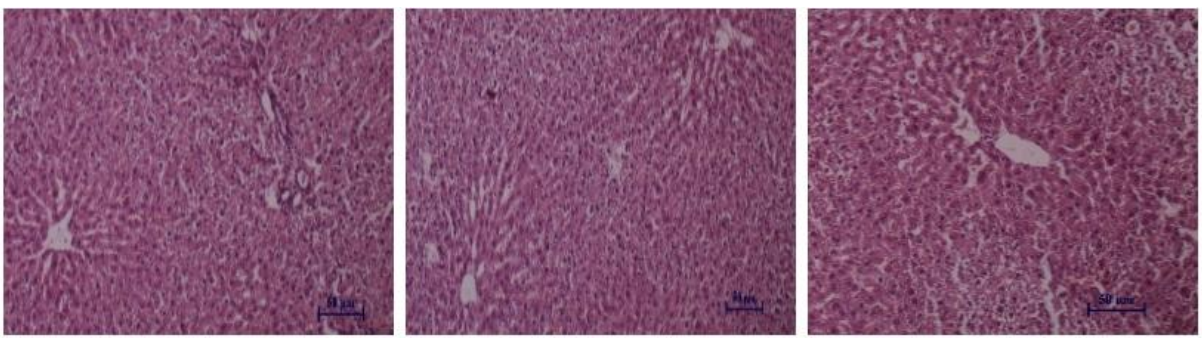


Figure 3.14.The effect of AuNPI3Cs on serum cholesterol of Swiss albino mice. Values are expressed as Mean ± SEM; n=6 for each group. Analysis was performed by one way ANOVA.

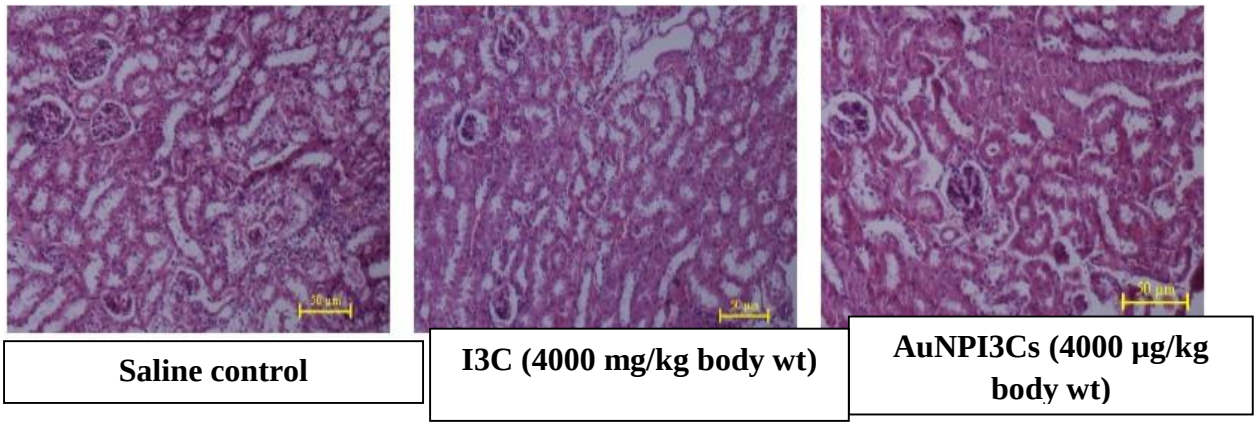
3.3.4.4 Effects of AuNPI3Cs on histopathology

Light microscopic examinations of the transverse section of vital organs i.e. kidney, liver of the control and AuNPI3Cs treated group mice are shown in Figure 3.15. Histopathological examination revealed that there was no change in structure of treated groups compared to control and also any gross pathological lesion was not observed in treated group when compared with control.

A



B



Saline control	I3C (4000 mg/kg body wt)	AuNPI3Cs (4000 µg/kg body wt)

Figure 3.15.Photomicrograph of the liver (A), kidney (B) section of mice administered with AuNPI3Cs at different doses for 28 days.

3.3 Discussion

Better and convenient methods have to be developed to evaluate the toxic effects of nanomaterials. Till now, few studies have reported the toxic effect of nanomaterials on *A. salina* (Ates et al., 2013; Pretti et al., 2014). On the other hand, the brine shrimp lethality assay has been used in toxicology research (Nunes et al., 2006).

In this study it was revealed that AuNPI3Cs exhibited concentration dependent cytotoxicity against brine shrimp. Bio assay lethality test obtained LC_{50} value of AuNPI3Cs was 2500 μgml^{-1}. Comet assay of brine shrimp found that AuNPI3Cs did not induce DNA damage compared to control group. Artemia has several advantages that make it perfect for toxicity assays (Nunes et al., 2006). The brine shrimp assay is suitable because it is rapid and simple. The eggs of *A. salina* are always available at low cost and remain viable for years.

AuNPI3Cs used in this study exhibited remarkable stability and uniform dispersion. No agglomeration and precipitation were observed. Zebrafish is a simple model for assessment of toxicity of any nanoparticles. Zebrafish is very useful for toxicity testing because its genome has some similarity with human genome. In this study no significant morphological

alteration in embryonic development was seen. But in high concentration of AuNPI3Cs (3000 $\mu g ml^{-1}$) zebrafish embryo showed some toxic effects like edema in thoracic cavity (Bonnie et al., 2017). It can be concluded that AuNPI3Cs do not possess harmful effect on zebrafish embryos.

GSH and MDA level of human lymphocytes were not altered significantly in AuNPI3Cs treated group compared to lymphocyte control group. ROS generation is a principal mechanism leading to nanotoxicity, including DNA damage, unregulated cell signaling, cytotoxicity, apoptosis, and cancer initiation and promotion (Fu et al., 2014). It was also revealed that ROS generation was not occurred in AuNPI3Cs treated lymphocytes compared to control lymphocytes. Flow cytometry study also revealed that cell death was not occurred in treated lymphocytes. DNA damage was not observed in treated group. So, it was confirmed that AuNPI3Cs exhibited no cytotoxic and genotoxic effect on human lymphocytes.

In general, the safety studies of any drug have been carried out by performing acute and sub-acute toxicity assays in laboratory animals (Fennell et al., 2004). Therefore, the aim of the present study was to evaluate the sub-acute toxicity study of AuNPI3Cs. The sub-acute toxicity study of AuNPI3Cs was performed on Swiss albino mice at different dose levels of 50, 100, 250, 500, 2000, 4000 $\mu g\ kg^{-1}$ body weight whereas the dose levels of I3C were 50, 100, 250, 500, 2000, 4000 $mg\ kg^{-1}$ body weight.

In sub-acute toxicity study, six groups of mice were treated of I3C and AuNPI3Cs for 28 days and the effects were monitored on biochemical and hematological parameters and histopathological features of liver and kidneys of treated animals. Interestingly, none of the studied parameters showed any evidence of adverse effects up to 4000 µg/kg treated group of mice.

The relative weight of vital organs like liver, kidney, heart and spleen were normal which indicates that treated group had no toxic effects. The safety profile of AuNPI3Cs is supported by the absence of any significant difference in liver, kidney, heart, spleen weight. After treatment of AuNPI3Cs for 28 days, the haematological parameters had no significant change ($p<0.001$) up to dose level of 4000 µg/kg body weight compared to control. Haematopoetic system are prone to have toxic effect of compounds (Kifayatullah et al., 2015). It may be mentioned that the rapid rate of regeneration of hematopoiesis has been considered as a subtle goal for toxicity testing (d'Yvoire et al., 2012).

For toxicity testing of any new compound, liver and kidney function is an important factor. Serum urea and creatinine level were not changed up to the dose level of 4000 µg/kg body weight of AuNPI3Cs compared to saline control group. Since, ALT, a hepatic cytoplasmic enzyme, when found in very high concentration, suggests possible hepatocellular damage. This incident was also seen in the study of Rhiouani et al. (Rhiouani et al., 2008). There was no significant variation of ALP, GOT and GPT up to the dose level of 4000 µg/kg body weight of AuNPI3Cs. Serum ALP levels are generally increased in case of liver diseases (Brautbar and Williams, 2002). AuNPI3Cs does not affect liver function after sub-acute administration in mice.

Another important finding of this study was that the histological assessments of liver and kidneys of animals that received AuNPI3Cs for 28 days showed no alteration in liver and kidney. All these findings documented in this study firmly demonstrated that AuNPI3Cs did not alter the liver or renal function which indicates the non-toxic nature of AuNPI3Cs.

3.4 Conclusion

The present study concluded that AuNPI3Cs are non-toxic up to the dose of 3000µg ml^{-1} for zebrafish and brine shrimp. The sub-acute toxicity study revealed that AuNPI3Cs did not

produce adverse effects on liver and kidney in mice up to the dose of 4000µg/kg body weight for 28 days. Hence, the present study proposes that AuNPI3Cs should be considered as safe for use up to 4000µg/kg body weight.