*Chapter 4*

# Toxicity assessment study between chemical and green synthesized Copper Oxide Nanoparticles

**INTRODUCTION:**

Copper oxide nanoparticles (CuONPs) are extensively used in semiconductor devices, solar energy converter, batteries, microelectronics, gas sensor and heat transfer fluids and are also used in manufacturing processes as industrial catalysts (Jiang and Zhang, 2010; Song et al.,2010). Further, CuONPs have attracted attention in many biomedical applications mostly due to their antimicrobial activity against a wide range of pathogenic microorganisms and cost effective synthesis (Perreault et al., 2012).

Despite its great potential in biomedical applications, toxicity studies of CuONPs reveals its cytotoxic effect in human airway epithelial cells (Fahmy and Cormier, 2009) and smooth muscle cells (Berntsen et al., 2010) rendered by oxidative stress. CuONPs induced $P^{38}$ has been shown to phosphorylate in mice endothelial cells due to oxidative stress which upregulated the plasminogen activator inhibitor-1 (Yu et al., 2010) and elicits DNA damage and apoptosis (Ahamed et al., 2008). Furthermore, synthesis of CuONPs by thermal decomposition (Salavati-Niasari and Davar, 2009), containing toxic chemicals, high temperature and high pressure is responsible for environmental toxicity and in addition is toxic to living systems (Nasrollahzadeh, M. 2014).

To reduce the toxicity on normal cells, green synthesized CuONPs* (S2NPs) has surfaced as a biocompatible alternative *prima facie* attributing to its biological accessibility to the target organ through biological barriers (Perreault et al., 2012). Biogenic synthesis of metal NPs being easy can be produced in a large scale without any contamination and also provides distinct morphology of the particles (Hutchison, JE. 2008). S2NPs possesses several advantages and demonstrates better efficacy compared to physical or chemical synthesis method (Nasrollahzadeh et al., 2015; Dubey et al., 2010). Among various plants, *A. indica*, a traditional medicinal plant which grows mainly in tropical and semi-tropical climates have been found to have versatile applications in medical science (Puri, HS. 1999). The leaves, flowers, fruits and seeds of *A. indica* have promising chemo preventive and therapeutic properties (Morgan, ED. 2009). It has also been reported that components of *A. indica* suppress NF-κβ signalling pathways (Priyadarsini et al., 2010).

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Van der Waals and electrostatic forces responsible for cellular internalization in $Cu^{+2}$ O ions, facilitate adhesive interaction of the CuONPs with the cell surface, thereby promoting cellular uptake of the NPs (Geiser et al., 2005).

*In vitro* toxicity of CuONPs depends not on the Cu release in the growth medium but on the NPs direct penetration into cells and subsequent dissolution of NPs, followed by distribution of toxic $Cu^{+2}$ ions into the cytoplasm of cell (Midander et al., 2009; Studer et al., 2010).

To my current knowledge, detailed report regarding the toxicity and biodistribution of green synthesized CuONPs *in vitro* and *in vivo* has been eluded. Hence, for further practical implementations, it is essential to evaluate the *in vitro* and *in vivo* toxicity of CuONPs and their biodistribution for the purpose of risk comprehension.

Toxic effect on several organs and lymphocytes was investigated. The current study is designed to evaluate biodistribution and toxicokinetics using green and chemical CuONPs synthesized in our laboratory (Dey et al., 2019; Ghosh et al., 2011).

Herein a comparative *in vitro* toxicity study of chemical CuONPs with green CuONPs in human lymphocytes by investigating the Cu ions internalization, biochemical estimation, ROS generation and apoptotic study was conducted. In addition, the *in vivo* toxicity of Chemical CuONPs (S1) and green CuONPs (S2) by evaluating biochemical parameters, apoptotic and cytokines estimation was investigated. The histopathology of different tissues after 15 days repeated intraperitoneal injection in Balb/c mice was also studied.

Furthermore body weight, organ weight, organ distribution and excretion to elucidate the primary accumulation sites and elimination routes of chemical and green CuONPs *in vivo* were investigated. In this work, for the best of my knowledge, the biodistribution and detailed toxicity of S1NPs and S2NPs *in vivo* by conducting a repeated dose toxicity study is reported.

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## 4.1. MATERIALS AND METHODS:

### 4.1.1 Chemicals and Reagents:

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetra-zolium bromide (MTT reagent), ethidium bromide, acridine orange and Histopaque-1077 were procured from Sigma (St. Louis, MO, USA). RPMI 1640, fetal bovine serum (FBS), sodium chloride (NaCl), penicillin, streptomycin, sucrose, sodium carbonate ($Na_2CO_3$), ethylene di-amine tetra acetate (EDTA) and dimethyl sulfoxide (DMSO) were purchased from Himedia, India. Tris-HCl, Tris buffer, $KH_2PO4$, $K_2HPO4$, formaldehyde, alcohol and all other chemicals of the highest purity grade were procured from Merck Ltd., Mumbai, India.

### 4.1.2. Selection of Human subjects and Isolation of Peripheral blood Lymphocytes:

Lymphocytes of six healthy human subjects devoid of any hereditary disease, chronic disease, drug addiction and medications were obtained. The human subjects belonged to the same geographical area and underwent regular routine checkup. The study protocol approved by the Ethical committee of Vidyasagar University (Approval No. IEC/6-20(Mod)/C-10/16), was in agreement with the declaration of Helsinki, as also previously reported from our laboratory (Chattopadhyay et al., 2013).

In compliance with Hudson and Hay (1989) blood samples were collected from six healthy human subjects in 5mL heparin coated vacutainers using veni-puncture method. After diluting 5mL blood 1:1 with phosphate buffered saline (PBS), Histopaque 1077 (Sigma) was used for density gradient centrifugation at 400g (1500rpm) for 40min at room temperature using a Pasteur pipette. Lymphocytes comprising the upper monolayer of buffy coat were collected using a pipette and washed thrice in balanced salt solution. Supplemented with 10% FBS, the peripheral blood lymphocytes (PBL) were re-suspended in RPMI complete media and incubated for 24h in a 95% air 5% $CO_2$ atmosphere at 37$^oC$ in $CO_2$ incubator.

### 4.1.3. Cell Culture:

Normal lymphocytes were cultured in a RPMI 1640 complete medium with 10% FBS, 2mM/L glutamine, 100U/mL penicillin, 100µg/mL streptomycin under 5% $CO_2$ and 95% humidified atmosphere at 37°C in $CO_2$ incubator.

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### 4.1.4. Drug Preparation:

Drug was prepared by making suspension of 10mg of S1NPs, S2NPs and Doxorubicin (DOX) in PBS. The working concentration (1, 5, 10, 25, 50 and 100µg/mL) of the drug was prepared by diluting the stock solution with PBS.

### 4.1.5. Toxicity *in vitro*:

#### 4.1.5.1. Toxicity of lymphocytes and Red Blood Cell (RBC):

Human lymphocytes ($2x10^5$ numbers of cells in each group) were plated into 96 wells of tissue culture plate and, after adding Doxorubicin and NPs (S1NPs and/or S2NPs) to the cells at varied concentrations (1, 5, 10, 25, 50 and l00µg/mL), were incubated for 24hr at 37°C in a humidified incubator (NBS) maintained with 5% $CO_2$. After 24hr the cell viability was estimated by adding 3-(4,5-dimethyl-thiazol)-2-diphenyltetrazolium bromide (MTT) and kept it in incubator for 2hr 15 mins as previously reported (Chattopadhyay et al., 2015). Then DMSO was added and after 30 min incubation optical density (ELISA microplate reader;Bio-rad, India) was measured.

RBC toxicity was estimated through hemolysis assay as previously reported (Chattopadhyay et al., 2015). 5mL of EDTA-stabilized human blood samples obtained from healthy subjects were added to 10mL of PBS, followed by centrifugation at 2200 rpm for 10min to obtain RBCs. After washing three times with 10mL of PBS solution, the RBCs were added to 50mL with PBS. For the positive and negative controls, RBCs were incubated with deionized water and PBS and 0.2mL of diluted RBC suspension obtained was mixed gently with 0.8mL of S1NPs and/or S2NPs solutions at varied concentrations. The mixtures were kept at room temperature for 3hr, centrifuged at 10000rpm for 3min, and subsequently supernatant (100µL) from all samples were taken to a 96-well plate. The absorbance values of the supernatants at 570nm were estimated using an ELISA microplate reader (Bio-rad, India) with the absorbance at 655nm as a standard. The percent hemolysis of RBCs was determined using the following formula:

Percent of hemolysis = (sample absorbance - negative control absorbance)/ (positive control absorbance - negative control absorbance) x 100

#### 4.1.5.2. Intracellular concentration of NPs:

The concentrations of Cu ions inside the cells were estimated by AAS. The lymphocytes were treated with 100µg/mL dose of both S1NPs and S2NPs for different durations (0, 12, 24 and 48hr). Acid digested samples were used for the measurement of Cu ions inside the cells using

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Shimadzu AA-7000 atomic absorption spectroscopy in RPMI 1640 medium as previously reported (Chattopadhyay et al., 2015). A typical reference curve of six standard samples (0.001, 0.0005, 0.00025, 0.00001, 0.000005 and 0.000001 M) was prepared using $CuSO_4.5H_2O$, dissolved in RPMI media.

**4.1.5.3. Biochemical Toxicity markers:**

After the isolation from the blood, the lymphocytes of different groups, the NADPH oxidase activity from the supernatant was evaluated spectrophotometrically by measuring cytochrome c reduction at 550nm (Heyneman and Vercauteren, 1984).

Estimating of Lactate Dehydrogenase (LDH) using a sandwich ELISA Kit (Tulip, Mumbai, India) and expressed as mg/dl. After the treatment (24hr), cell supernatant was used for LDH measurement as per the detailed instructions of the manufacturer.

From the supernatant of cell lysate, lipid peroxidation level was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA). Cell supernatant and distilled water taken at a ratio of 1:1 and added 8.1% sodium dodecyl sulfate (SDS) which was half volume of cell supernatant. Subsequently 375µl of both 20% acetic acid ($p^H$ 3.5) and thiobarbituric acid (0.6%) were added to the tissue solution and placed in a boiling water bath for 1h. After boiling, double distilled water (250µl) along with 15:1 butanol–pyridine solution (1.25mL) were added to the mixture and subsequently centrifuged at 2000g for 5min. The supernatant was removed and MDA concentrations were measured spectrophotometrically at 530nm using Hitachi U-2000 spectrophotometer. The MDA levels were expressed as nmol/mg protein (Mahapatra et al., 2009).

Nitric Oxide (NO) release assay was performed in accordance with Chakraborty et al. (2011). NO values were expressed as µM/mg protein.

**4.1.5.4. Intracellular ROS generation:**

Intracellular ROS estimation was performed using 2,7-dichlorofluorescein diacetate (DCFH2-DA) as previously reported using fluorescence microscopy (Nikon ECLIPSE LV100POL). Fluorescence was measured by transferring a 1mL supernatant to a cuvette at 520nm emission and 485nm excitation using a fluorescence spectrophotometer (HitachiF-7000, Singapore). The

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values were expressed in terms of percent of fluorescence intensity relative to the control wells (Chattopadhyay et al., 2015).

#### 4.1.5.5. Apoptotic or necrotic event analysis:

Cellular morphology was visualized using ethidium bromide (EtBr) in combination with acridine orange (AO) staining by EtBr/AO double staining. Etbr/AO double staining is a vital process for investigating the toxicity of S1NPs and S2NPs. Etbr stains the nuclear changes and AO is used to detect the apoptotic body formation inside the cytoplasm. Upon using both EtBr and AO together, green color obtained indicates living cells, whereas orange and red color indicates late apoptosis and necrosis respectively. After treatment of cells with S1Ps and S2NPs, the lymphocytes ($2\times10^5$cells/mL) were washed with cold PBS and then stained solution of PBS containing EtBr and AO (50µg/mL; Vol/Vol) at room temperature for 5 min. Subsequently, the cells were washed thrice with PBS, and images of the stained cells were observed under fluorescence microscope (NIKON ECLIPSE LV100POL) at 40X magnification (Das et al., 2017).

#### 4.1.5.6. Cytokines analysis:

Cytokines including pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) were measured using ELISA assay kit (Enzyme linked immunosorbent assay). Pre coated plates of Human TNF-α ELISA Ready-SET-Go, E-bioscience, India were used in accord with manufacturer's instruction. The sensitivity limit of the cytokines was 4.0pg/mL and 2.0pg/mL for TNF-α and IL-10, respectively. The concentrations of the cytokines were expressed as pg/mL/$10^6$ cells.

#### 4.1.5.7. Pro and Anti-apoptotic marker analysis:

The level of pro-apoptotic factors (Caspase-8, Caspase-3, p38 and Caspase-9) and anti-apoptotic factors (pAKT and Bcl2) were estimated using an ELISA (Akiyama et al., 2012). Optical densities were determined at 450nm using an ELISA reader (BioRad) and all the experiments were performed thrice. The supernatant of lymphocytes without any treatment was used as positive control.

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## 4.2. *In vivo* toxicity assessment:

### 4.2.1. Animal maintainance:

Female Balb/c mice of weight between 25-35gm were taken for the experiment. The age of the mice was 6-8 weeks old. Standard vitamin rich pellet diet, water *ad libitum* was provided to them. Polypropylene cage (Terson) was used. Paddy husk was embedded at the bottom of cage. They were kept in the departmental animal house with a maintained 12hr light & dark cycle under room temperature. According to the guideline of the National Institute of Nutrition, Hyderabad, India and Indian Council of Medical Research and the approval by the ethical committee of Vidyasagar University (approval no IEC/6-10(Mod)/C-9/16.) mice were maintained. Balb/c mice were divided into five groups, containing six mice each. After S1NPs and S2NPs were suspended in a PBS solution of $p^H$ 7.4, both NPs were ultra sonicated for 10min and subsequently injected intraperitoneally with 100, 200, 500 and 1000µg/kg body weight doses. The mice were injected at every 3 days interval for 15 days (Chattopadhyay et al., 2015).

### 4.2.2. Assessment of Serum Chemistry:

Blood sample of the Balb/c mice was kept at room temperature for 2hr, followed by centrifugation at 850g for 15min. using the serum obtained LDH, SGOT and creatinine levels were ascertained according to the kit manufacturer's instruction.

### 4.2.3. Euthanasia of experimental animals:

When the experimental treatment was completed, all the mice were euthanized by cervical dislocation under ketamine-xylazine anaesthesia. Before euthanasia mice were deprived of food. After the dissection of mice, the organs were weighed and homogenized for the further experiments.

### 4.2.4. Quantitative analysis of S1NPs and S2NPs in Tissues:

Around 0.1-0.5g tissue (Liver, Lungs, Kidney, Spleen, Heart, Intestine and feces) and almost 0.3mL of blood sample was allocated to determine the amount of Cu ions of S1NPs and S2NPs using AAS (Yang et al., 2017).

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### 4.2.5. Estimation of cytokines level and Pro and anti-apoptotic markers analysis from Serum:

Cytokines estimation and Apoptotic markers estimation were performed according to above mentioned method in section 4.1.5.6 and 4.1.5.7. The serum of Control mice was used as a positive control.

### 4.2.6. Histopathological study of tissues:

Tissues were fixed in 10% formalin, followed by embedding in paraffin. Subsequently, 5µm thick paraffin section was obtained and stained with hematoxylin and eosin (Harrison et al., 2010). Light microscope was used to observe any tissue impairment.

### 4.2.7. Estimation of Protein:

Protein content was analyzed from cell free supernatant of lymphocytes. Protein estimation was performed in accordance with Lowry et al. (1951).

### 4.2.8. Statistical analysis:

Entire data were represented as mean ± SEM. Means of two groups were analogized using two-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with several comparison t-tests. $p < 0.05$ was taken as a limit of significance.

## 4.3. RESULTS:

### 4.3.1. *In vitro* study:

#### 4.3.1.1. Cell viability of Lymphocytes:

As shown in **Fig. 4.1**, both S1 and S2NPs significantly reduced lymphocytes at both time points compared to the control. Percentages of cell death increased likewise with increment in doses (1-100µg/mL) for both NPs. However, after 24h, percentages of cell death in S1NPs treated lymphocytes (19.57%, 31.34%, 45.77%, 61.64%, 74.54% and 85.69%) were significantly higher compared to control than that of S2NPs treated lymphocytes (12.56%, 21.94%, 32.82%, 47.00% ,65.11% and 79.77%) at similar doses. Additionally, DOX killed lymphocytes by 25.71%, 57.74%, 69.3%, 75.69%, 89.30% and 94.03% compared to control after 24h. After 48hr the % of cell death increased in case of both NPs and DOX in a dose dependent manner. But S1NPs killed lymphocytes more than S2NPs which indicate more toxic effect on lymphocytes. Hence on the

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basis of % of cell death, 24hr was taken into consideration for further treatment although it was also toxic but less than 48hr.

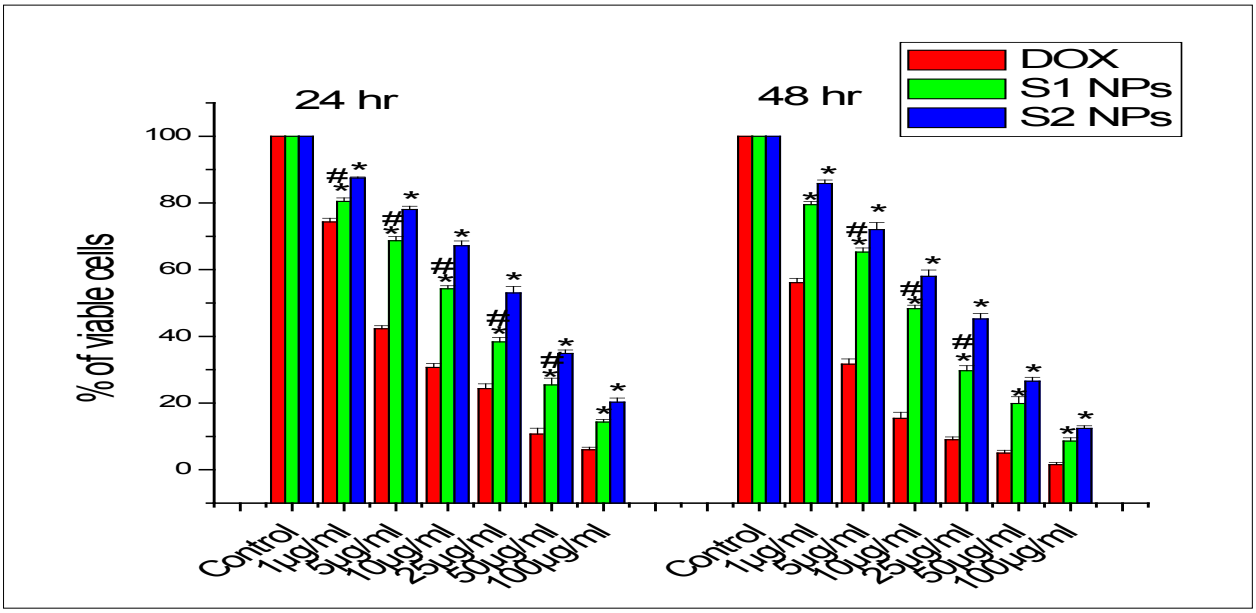


**Fig. 4.1:** Cell viability assay based on dose and duration dependent percentage of Lymphocytes death by S1NPs, S2NPs and DOX was estimated by MTT assay. n=3, values were expressed as mean±SEM. Superscripts indicated a significant difference as ($P<0.05$) compared with control.

### 4.3.1.2. Intracellular concentration of NPs in Lymphocytes:

As shown in Fig.4.2A, intracellular concentration of S1NPs and S2NPs for varied time points at 100µg/mL dose was observed. After 24hr, the Cu ions concentration in S1NPs treated lymphocytes (0.58pg ions/cell) was significantly higher than S2NPs treated lymphocytes (0.34pg ions/cell).

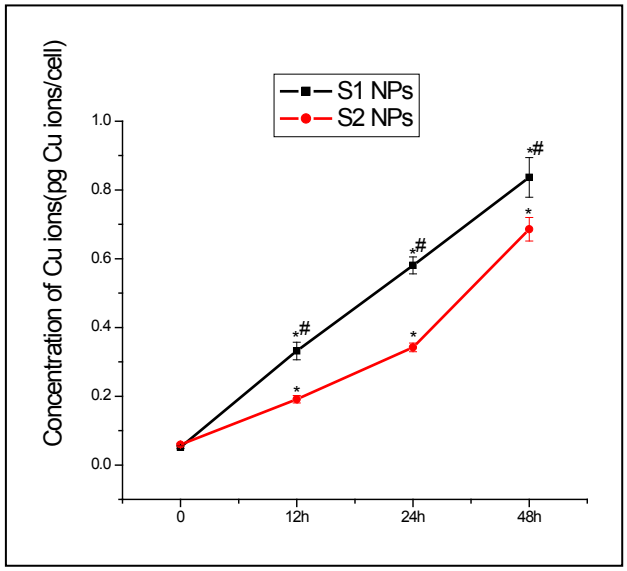


**Figure 4.2A:** Estimation of Cu ions concentration inside the lymphocytes after treatment with S1 and S2NPs at different time duration by AAS.

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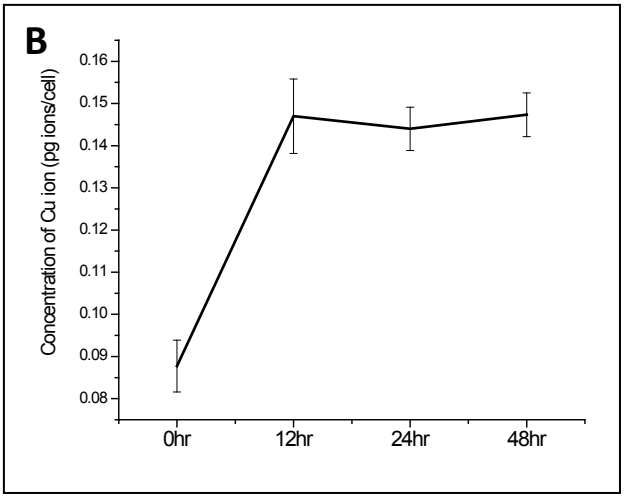


Intracellular concentrations of Cu ions in lymphocytes were 0.148pg ions/cell. That mean the (0.58-0.148) and (0.34-0.148) =0.432 and 0.192pg ions/cell was due to both the CuONPs.

**Figure 4.2B:** Estimation of Cu ions concentration inside the lymphocytes at different time duration by AAS. Here no treatment was done. Only lymphocytes were used to measure Cu ions.

### 4.3.1.3. Hemolysis assay:

The hemolytic activity of S1NPs and S2NPs exhibited increasing trends with increase in doses from 10-100µg/mL and 50-100µg/mL respectively compared to the negative control after 24hr. Though, notably the hemolytic activity of S1NPs (0.46, 0.52, 0.77 and 0.87g/dl) (Fig. 4.3A) was significantly higher compared to control than that of S2NPs (0.270, 0.292, 0.44 and 0.547g/dl) for 10-100µg/mL (Fig. 4.3B).

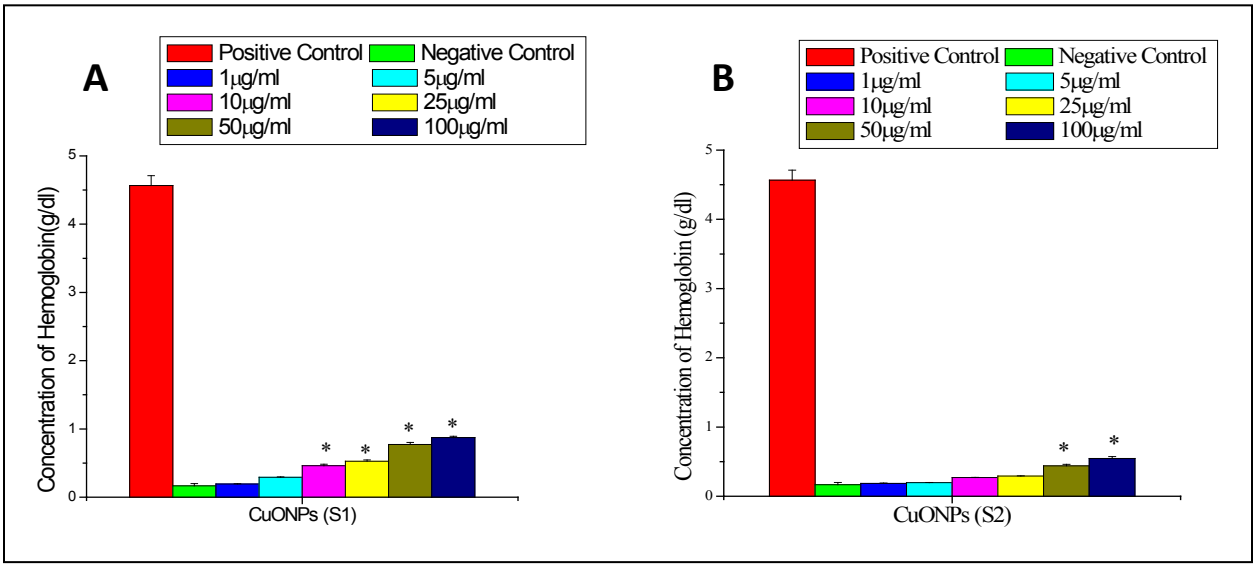


**Figure 4.3:** Estimation of hemolysis after **(A)** S1NPs and **(B)** S2NPs exposure for 24h. n=3,Values were expressed as mean±SEM. Superscripts indicated a significant difference as ($P<0.05$) compared with control.

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### 4.3.1.4. Biochemical assessment of Toxicity in Lymphocytes:

All the toxicity markers estimated from the cell lysate of DOX, S1NPs and S2NPs treated groups displayed increasing trends with gradual increase in dosages (1-100µg/mL) compared to control.

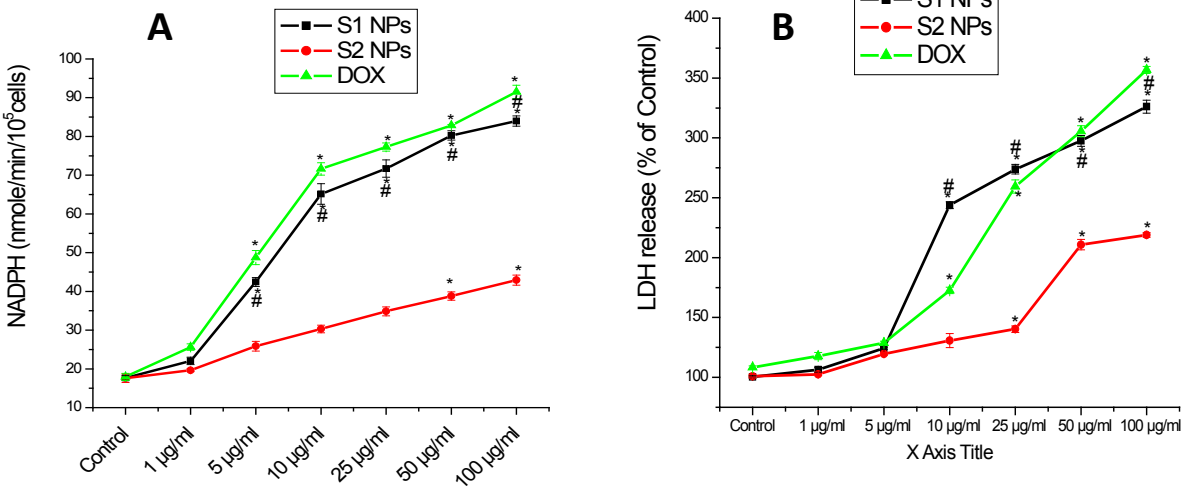


**Figure 4.4:** Estimation of **(A)** NADPH oxidase level, **(B)** LDH level from lymphocytes after treatment with S1NPs, S2NPs and DOX for 24hr. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant differences (P<0.05) compared with the control group and # superscripts indicate significant differences of S1NPs compared to S2NPs.

As shown in Fig. 4.4A, the NADPH oxidase activity of S1NPs (1.24, 2.40, 3.68, 4.05, 4.54, 4.75 folds) was significantly higher compared to control than that of S2NPs (1.11, 1.46, 1.72, 1.98,

| Toxicity Markers | Different Doses(µg/ml) Values expressed as Fold Change | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 5 | 10 | 25 | 50 | 100 |
| NADPH (S1) | 1.24 | 2.40 | 3.68 | 4.05 | 4.54 | 4.75 |
| NADPH (S2) | 1.11 | 1.46 | 1.72 | 1.98 | 2.20 | 2.43 |
| Nitric Oxide (S1) | 1.07 | 1.86 | 2.66 | 3.22 | 3.97 | 4.31 |
| Nitric Oxide (S2) | 1.01 | 1.12 | 1.24 | 1.33 | 1.51 | 1.65 |
| MDA (S1) | 1.21 | 1.32 | 1.75 | 2.65 | 3.26 | 3.71 |
| MDA (S2) | 1.14 | 1.26 | 1.50 | 1.94 | 2.20 | 2.48 |
| LDH (S1) | 1.05 | 1.23 | 2.42 | 2.72 | 2.96 | 3.25 |
| LDH (S2) | 1.01 | 1.19 | 1.30 | 1.40 | 2.10 | 2.18 |

**Table 4.1:** Represents the change in different biochemical toxicity markers (NADPH, Nitric Oxide, MDA and LDH) after treatment with S1NPs and S2NPs with different doses (1-100µg/ml). Values were expressed as a change in fold compared to control.

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2.20, 2.43 folds) at similar doses. Similarly S1NPs displayed higher MDA levels (shown in Fig. 4.5A) compared to control than that of S2NPs at all respective doses. Note that (in Fig. 4.4B and 4.5B) LDH and NO generation levels of S1NPs were significantly higher than that of S2NPs for doses 10-100µg/mL and 5-100µg/mL respectively. The total fold changes were detailed in Table 4.1.

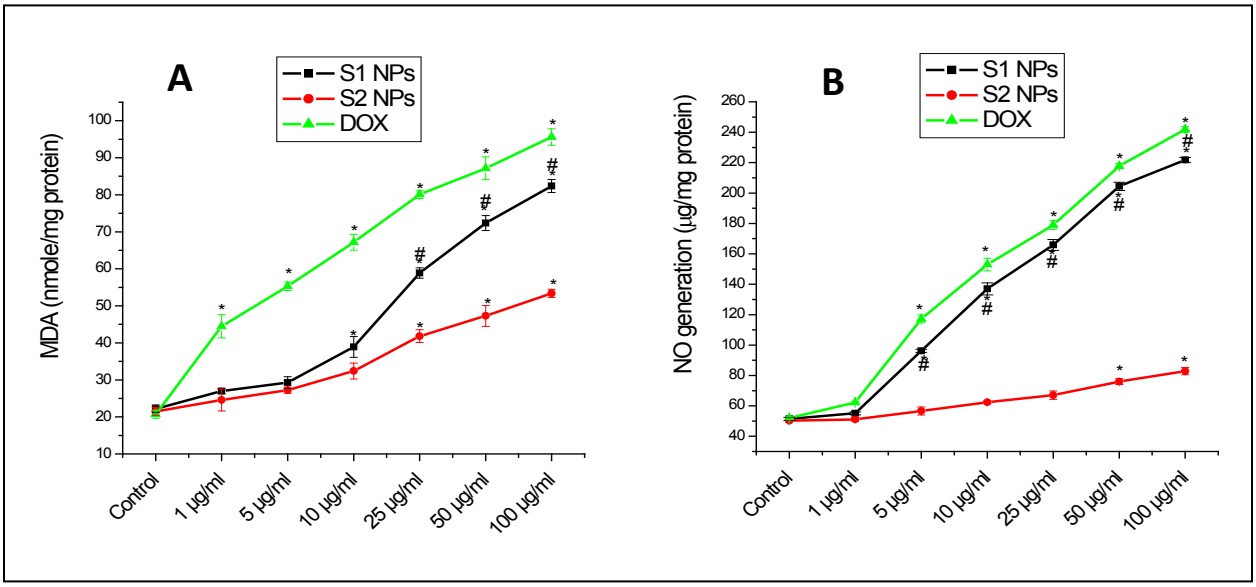


**Figure 4.5: (A)** MDA enzymatic level and **(B)** NO levels from lymphocytes after treatment with S1NPs, S2NPs and DOX for 24h. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant differences ($P<0.05$) compared with the control group and $^{\#}$superscripts indicate significant differences of S1NPs compared to S2NPs.

**4.3.1.5. ROS generation and Apoptotic or necrotic event analysis in Lymphocytes:**

As shown in **Fig. 4.6(A-B),** both S1NPs and S2NPs induced lymphocytes, displayed significant amount of ROS generation after 24h. Note that S1NPs showed increase in ROS generation gradually from initial to higher doses whereas S2NPs showed significant ROS generation only at higher doses (25-100µg/mL). Also at higher doses (25-100µg/mL), S1NPs displayed higher ROS generation compared to S2NPs, indicating higher oxidative stress of S1NPs.

From **Fig. 4.6(C-D),** significant apoptotic phenomenon in case of both the S1NPs and S2NPs was observed. S1NPs exhibited apoptotic event from 5-100µg/mL doses, whereas S2NPs displayed significant apoptosis from 25-100µg/mL doses. But notably S1NPs displayed more late apoptosis and degradation of lymphocytes shape compared to S2NPs. Note that both NPs did not exhibit necrosis in lymphocytes.

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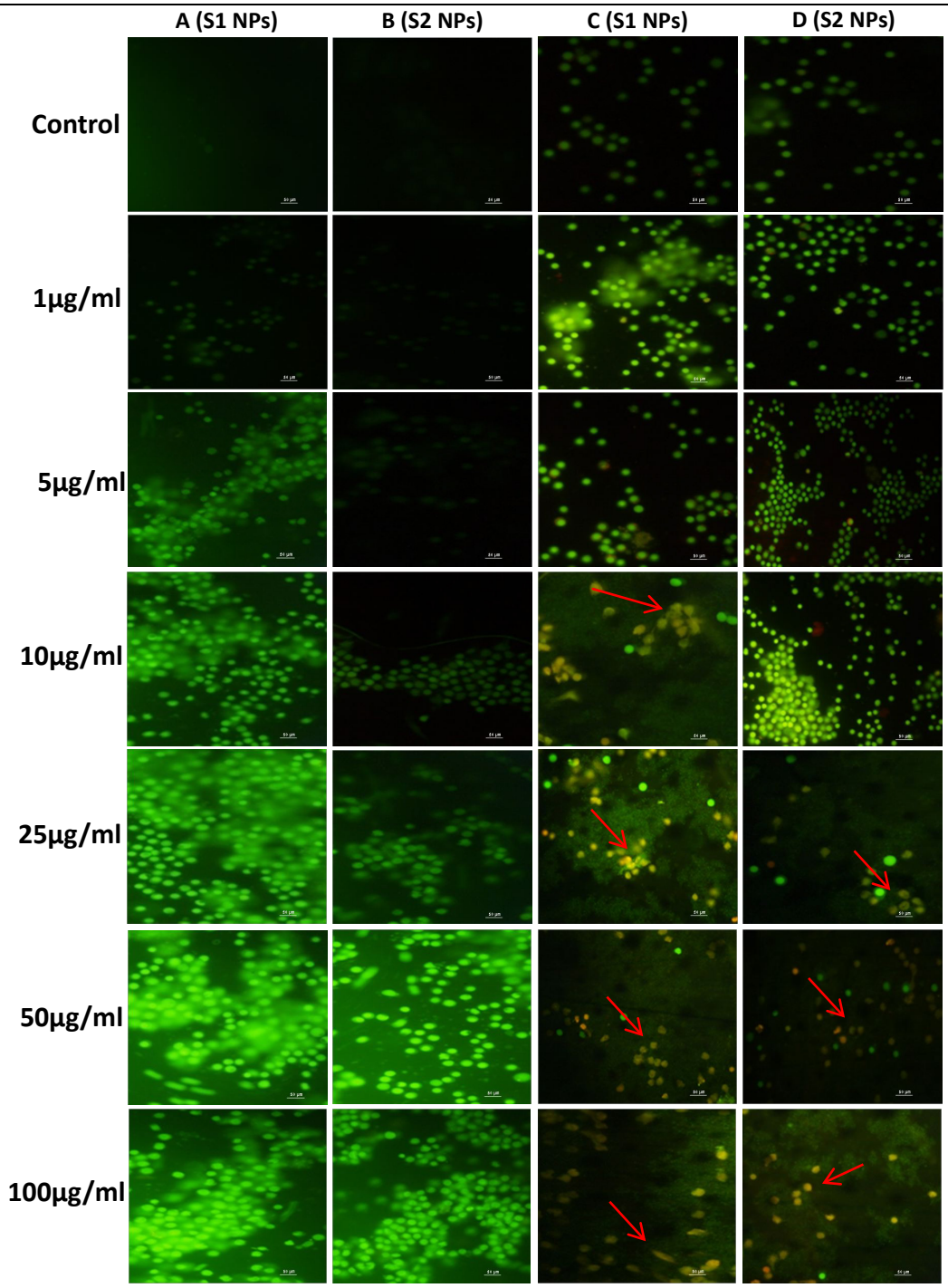


**Fig. 4.6:** Fluorescence microscopic images of ROS and Apoptotic phenomenon of human lymphocytes. **(A, B)** Effect of **(A)** S1NPs and **(B)** S2NPs on lymphocytes was visualized under fluorescence microscope by DCHF2DA staining with different doses. **(C, D)** Apoptotic or necrotic event of **(C)** S1NPs and **(D)** S2NPs on lymphocytes were visualized under fluorescence microscope by Etbr/AO double staining with different doses with original magnification of 400X.

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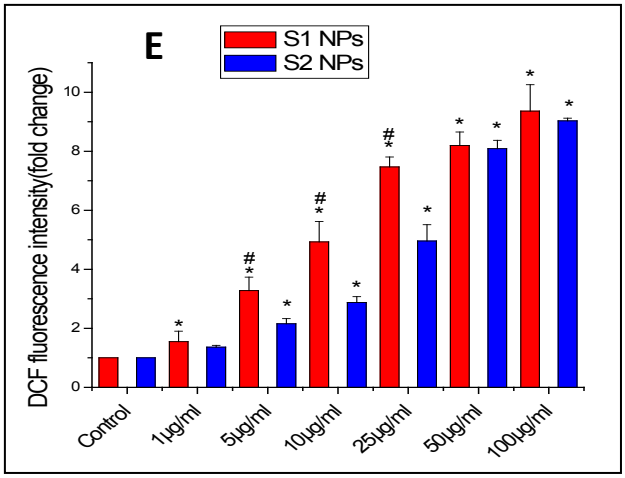


**Figure 4.6: (E)** Fluorescence intensity of DCF measured at 485nm excitation and 520nm emission using a fluorescence spectrophotometer. Intensity of control cells was set to 1.00. Data is represented as the fold change of the ROS level in the control group. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant difference (P<0.05) compared with the control group and # superscripts indicate significant differences of S1 NPs compared to S2 NPs.

### 4.3.1.6. Cytokines and apoptotic markers Analysis in Lymphocytes:

The pro inflammatory cytokine TNF-α level and anti- inflammatory cytokine IL-10 were altered significantly after S1NPs and S2NPs exposure in a dose-dependent manner (1-100µg/mL) compared to the control. S1NPs and S2NPs increased TNF-α levels by 1.08, 1.34, 1.91, 2.38, 3.14 and 3.68 folds and 1.03, 1.33, 1.45, 1.78, 2.29, 2.51 folds respectively. Contrarily anti-

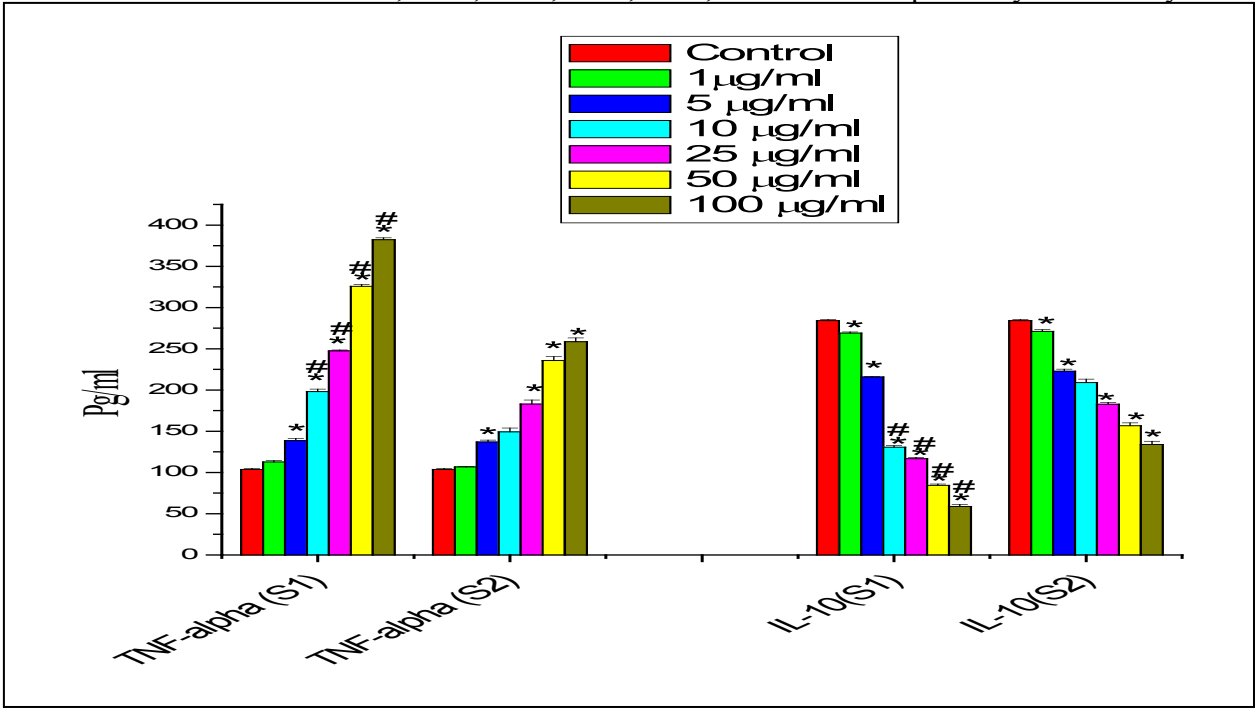


**Figure 4.7:** Cytokines analysis *in vitro*. Pro and anti-inflammatory response of S1NPs and S2NPs on lymphocytes after 24h treatment (TNF-α, IL-10). Values are expressed as mean±SEM of three experiments; *superscripts indicate significant differences (P<0.05) compared with the control group and # superscripts indicate significant differences of S1 NPs compared to S2 NPs.

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inflammatory cytokine IL-10 levels decreased 1.05, 1.31, 2.17, 2.43, 3.36, 4.83 folds and 1.03, 1.25, 1.33, 1.53, 1.78, 2.09 folds after treatment with S1NPs and S2NPs respectively **(Fig.4.7)**. Changes in pro and anti-apoptotic protein levels were estimated compared to the control group

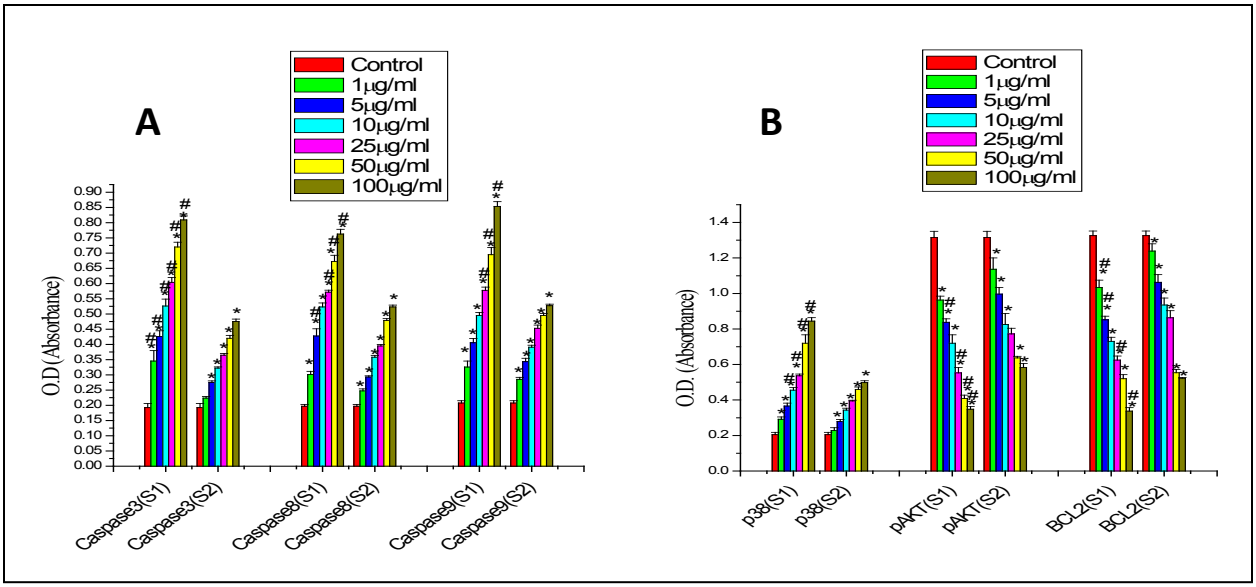


**Figure 4.8: (A, B)** Assessment of pro-apoptotic (Caspase-8, Caspase-9, Caspase-3, p38) and anti-apoptotic (pAKT, Bcl2) protein level after S1NPs and S2NPs treatment. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant differences ($P<0.05$) compared with the control group and $^{\#}$superscripts indicate significant differences of S1 NPs compared to S2 NPs.

from cell lysate using ELISA procedure. As shown in Fig. 4.8A**,** the pro-apoptotic proteins (Caspase-3, Caspase-8, Caspase-9, p38) levels were up-regulated, whereas the anti-apoptotic proteins (pAKT, Bcl2) expression levels were down-regulated after treatment with S1NPs and S2NPs with gradual increase in doses. However the pro-apoptotic proteins level increment in S1NPs was significantly higher compared to S2NPs at all respective dosages. The changes in protein expression levels were enumerated in Table 4.2, expressed in folds.

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| Apoptotic Markers | Different Doses (µg/ml). Values Expressed as Fold change | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 5 | 10 | 25 | 50 | 100 |
| Caspase 3(S1) | 1.78 | 2.12 | 2.73 | 3.52 | 3.78 | 4.21 |
| Caspase 8 (S1) | 1.52 | 2.16 | 2.65 | 2.89 | 3.41 | 3.86 |
| Caspase 9 (S1) | 1.57 | 1.95 | 2.39 | 2.78 | 3.35 | 4.12 |
| P38 (S1) | 1.42 | 1.78 | 2.23 | 2.62 | 3.52 | 4.13 |
| pAKT (S1) | 1.36 | 1.57 | 1.82 | 2.38 | 3.22 | 3.77 |
| Bcl2 (S1) | 1.28 | 1.55 | 1.81 | 2.12 | 2.55 | 3.93 |
| Caspase 3(S2) | 1.29 | 1.61 | 1.89 | 2.14 | 2.47 | 2.79 |
| Caspase 8 (S2) | 1.39 | 1.64 | 2.02 | 2.22 | 2.70 | 2.95 |
| Caspase 9 (S2) | 1.54 | 1.85 | 2.11 | 2.44 | 2.67 | 2.84 |
| P38 (S2) | 1.27 | 1.56 | 1.92 | 2.21 | 2.57 | 2.79 |
| pAKT (S2) | 1.15 | 1.25 | 1.39 | 1.55 | 2.24 | 2.40 |
| Bcl2 (S2) | 1.30 | 1.48 | 1.76 | 1.91 | 2.07 | 2.20 |

**Table 4.2:** Represents the change in different pro and anti-apoptotic markers level after the treatment with S1NPs and S2NPs with different doses (1-100µg/mL) on lymphocytes. Values were expressed as a change in fold compared to control

### 4.4.2. *In vivo* effects:

#### 4.4.2.1. Effects on Body weight and Organ Weight:

The body weight and organ weight of each animal were evaluated after 15days of treatment through i.p route at different dosages (100, 200, 500 and 1000µg/Kg) of bodyweight. As shown

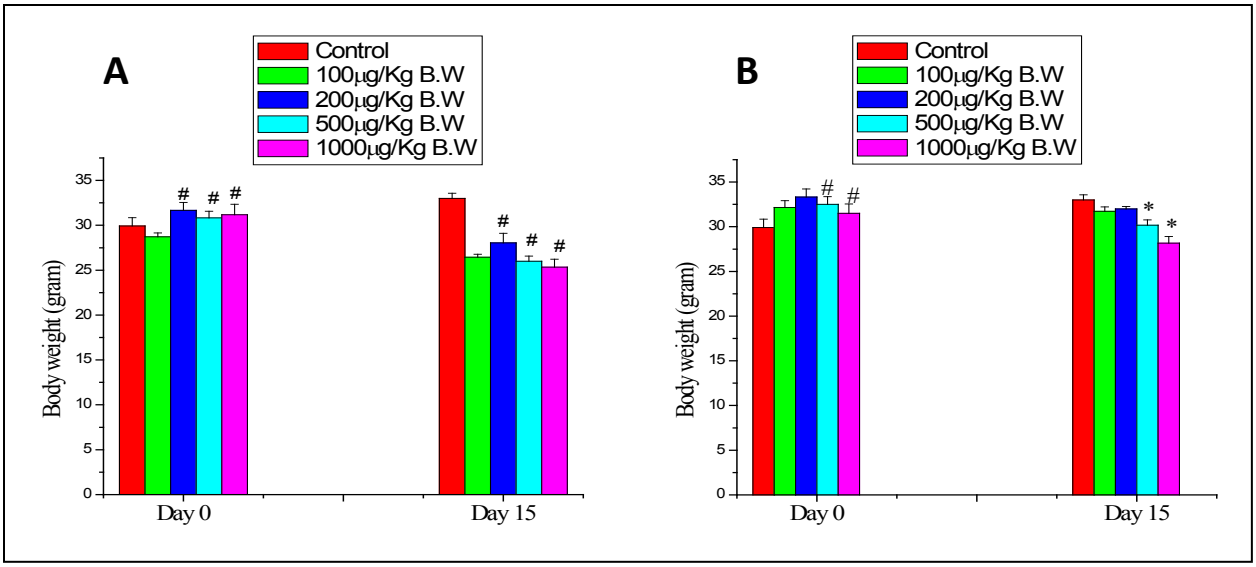


**Figure 4.9:** Estimation of body weight from animal **(A, B)** Estimation of body weight of **(A)** S1NPs and **(B)** S2NPs treated groups for 15 days. n=6; Values are expressed as mean±SEM; *superscripts indicate significant differences (P<0.05) compared with the control group

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in Fig. 4.9A, after 15 days, mice bodyweight of S1NPs treated group depleted significantly compared to the control group at all doses, suggesting toxicity of S1NPs. By contrast, bodyweight decreased in S2NPs experimental groups only at high doses (500-1000µg/Kg bodyweight) compared to control (Fig. 4.9B).

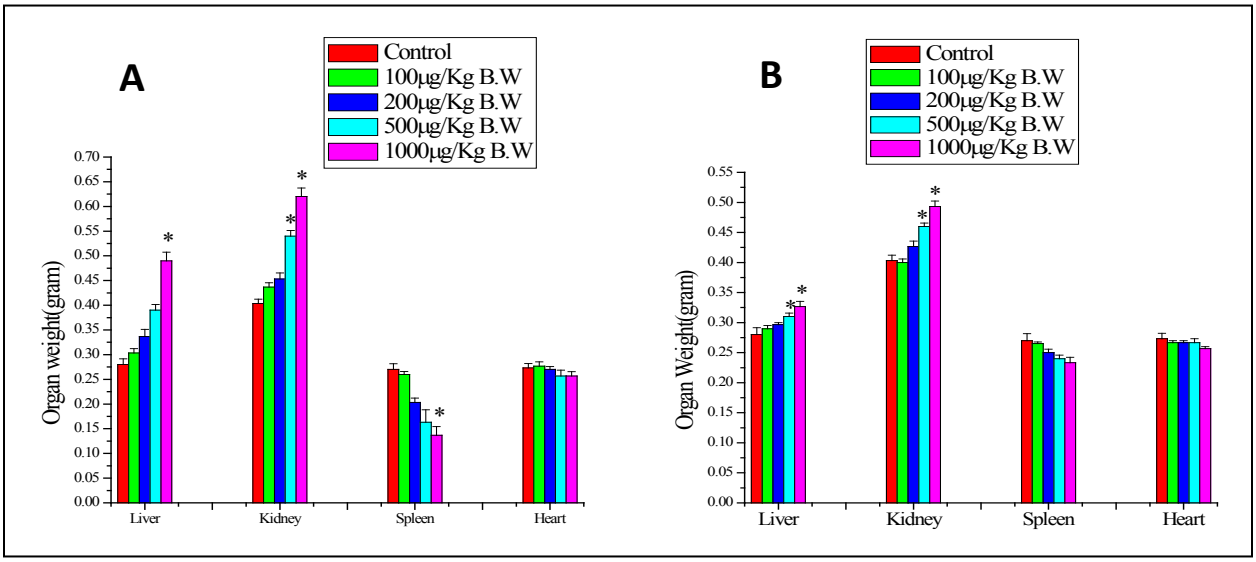


**Figure 4.10:** Estimation of organ weight. **(A, B)** Estimation of organ weight of **(A)** S1NPs and **(B)** S2NPs treated groups for 15 days. n=6; Values are expressed as mean±SEM; *superscripts indicate significant differences (P<0.05) compared with the control group

In Fig. 4.10A-B, liver and kidney of both experimental groups displayed similar upward trends in mass with increase in dosages (100-1000µg/Kg body weight) compared to control after 15 days. However, the increase in liver and kidney weight of S1NPs treated groups were considerably higher than those of S2NPs treated groups. The spleen weight (Fig. 4.10A-B) reduced significantly with increase in dosages compared to the control in both S1NPs and S2NPs treated mice, but the reduction was considerably higher in S1NPs treated groups. Organ weight changes are a significant indicator in toxicity studies. From the result, it can be assumed that both S1NPs and S2NPs exert toxic effects against liver, spleen and kidney. In heart, no significant changes in weight were observed in case of both experimental groups compared to the control.

### 4.4.2.2. Serum Chemistry:

Elevated LDH levels of serum were observed for dosages of 100-1000µg/Kg body weight in case of both S1NPs and S2NPs (Fig. 4.12). Furthermore**,** increment in SGOT and creatinine levels were also noted (Fig. 4.11A-B and C-D), thus indicating dysfunction of liver and kidneys. From

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Fig. 4.11 and 4.12 the values of LDH, SGOT and Creatinine were expressed in Table 4.3.

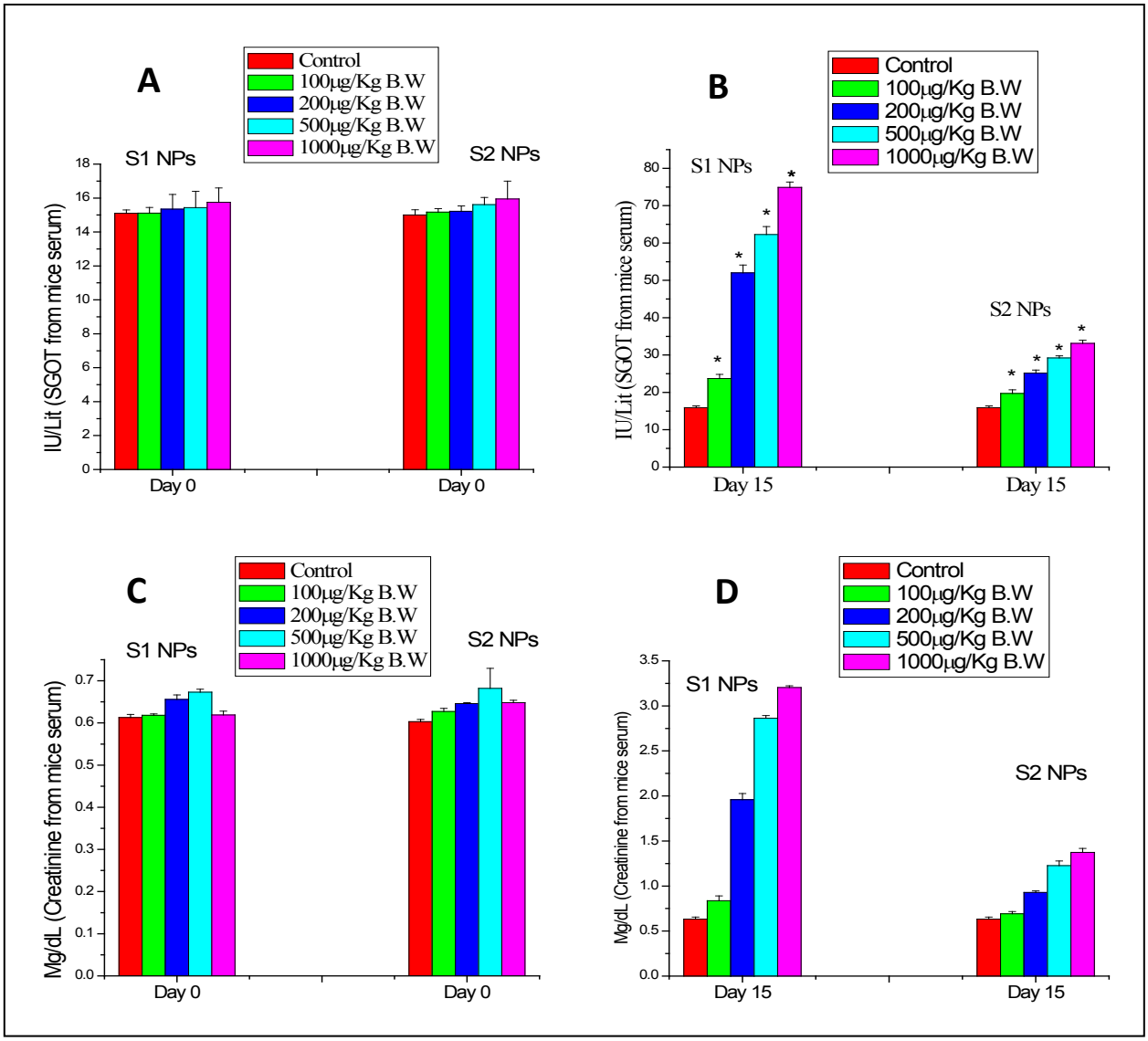


**Figure 4.11:** Estimation of **(A, B)** SGOT, **(C,D)** Creatinine from the serum of Balb/c mice after the treatment with S1NPs and S2NPs for 0 days and 15 days. Values are expressed as mean±SEM; n=6; *superscripts indicate significant differences ($P<0.05$) compared with the control group.

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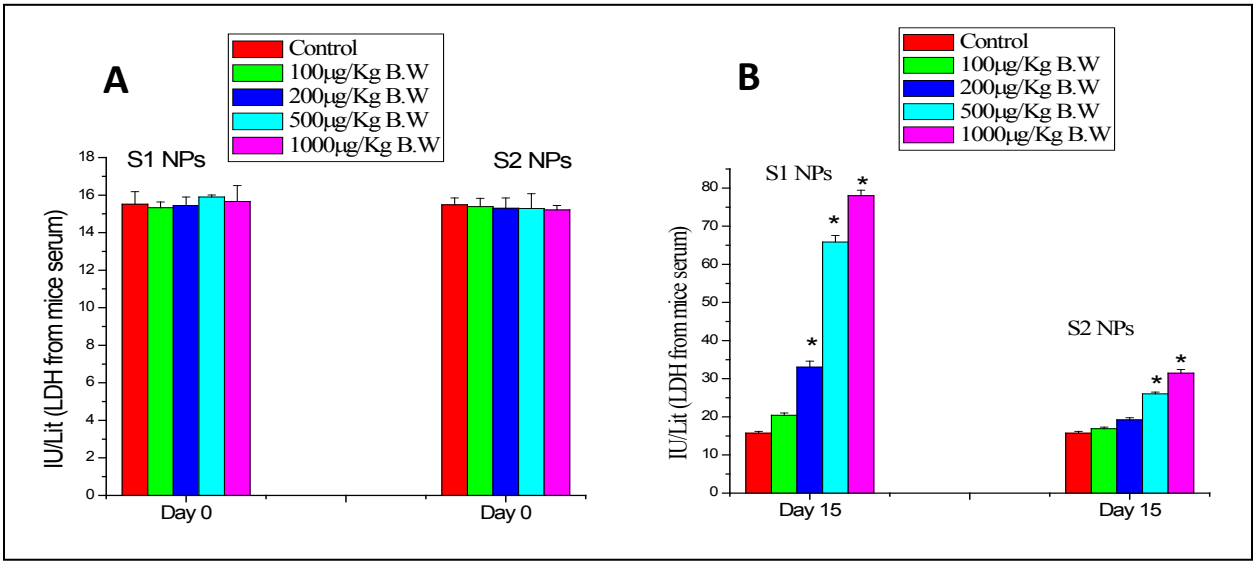


**Figure 4.12:** Estimation of **(A, B)** LDH levels from the serum of Balb/c mice after the treatment with S1NPs and S2NPs for 0 days and 15 days. Values are expressed as mean±SEM; n=6; *superscripts indicate significant differences (P<0.05) compared with the control group.

| Biochemical Markers from Mice serum | 15 Days Different Doses(µg/Kg body weight) | | | |
|---|---|---|---|---|
| | 100 | 200 | 500 | 1000 |
| LDH(S1) | 1.29 | 2.10 | 4.19 | 4.96 |
| LDH (S2) | 1.10 | 1.25 | 1.65 | 2.00 |
| SGOT (S1) | 1.49 | 3.27 | 3.91 | 4.71 |
| SGOT (S2) | 1.28 | 1.63 | 1.90 | 2.16 |
| Creatinine (S1) | 1.32 | 3.09 | 4.51 | 5.05 |
| Creatinine (S2) | 1.17 | 1.57 | 2.07 | 2.32 |

**Table 4.3:** Estimation of changes in biochemical markers (LDH, SGOT, Creatinine) from mice serum after treatment with S1NPs and S2NPs at different doses (100, 200, 500 and 1000µg/Kg body weight) for 15 days. Values were expressed as a change in fold compared to control.

### 4.4.2.3. Biodistribution and elimination of S1NPs and S2NPs:

As shown in (Fig. 4.13A-B), S1NPs and S2NPs were both accumulated in the liver and spleen, followed by the kidney, lungs, heart and intestine at the highest dosage of 1000µg/Kg body weight after 15 days. After 15 days S1NPs treated mice and S2 NPs treated mice showed 5.74

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folds and 2.74 folds of Cu ions accumulated in liver compared to the control group. Similarly significant amount of accumulation in spleen was also observed compared to the control group.

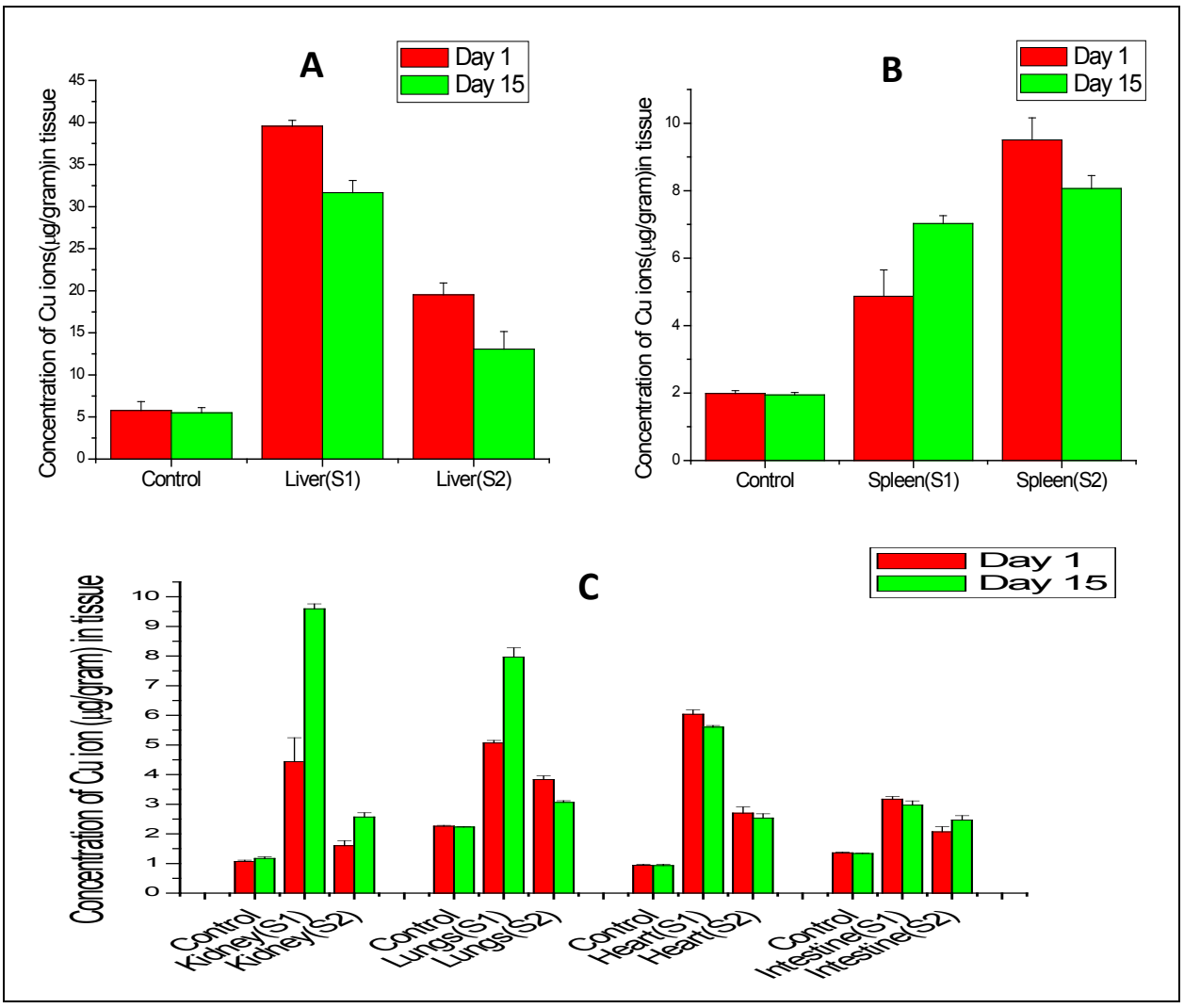


**Figure 4.13:** S1NPs or S2NPs levels in animal tissues as indicators of inorganic NPs biodistribution at 1000 µg/mL dose. **(A,B)** Exhibits the contents of **(A)** S1NPs or **(B)** S2NPs in the liver and spleen at both time points. **C)** Exhibits the contents of S1NPs or S2NPs in the other organs at both time points. n=6.

The S2NPs concentrations were significantly 2.02 folds (1 day) and 2.42 folds (15 days) lower compared to S1NPs concentrations in the liver, and in the spleen S2NPs concentrations were significantly 1.95 folds (1 day) and 1.14 folds (15 days) higher compared to S1NPs concentrations (Fig. 4.13**)**. However, concentrations of S1NPs showed 2.75 folds and 3.8 folds higher levels in the kidney compared with S2NPs (Fig. 4.13**)** at both time points. Moreover,

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concentrations of S1NPs were also significantly higher than the concentrations of S2NPs in the lungs, heart and intestine at both time points (Fig. 4.13).

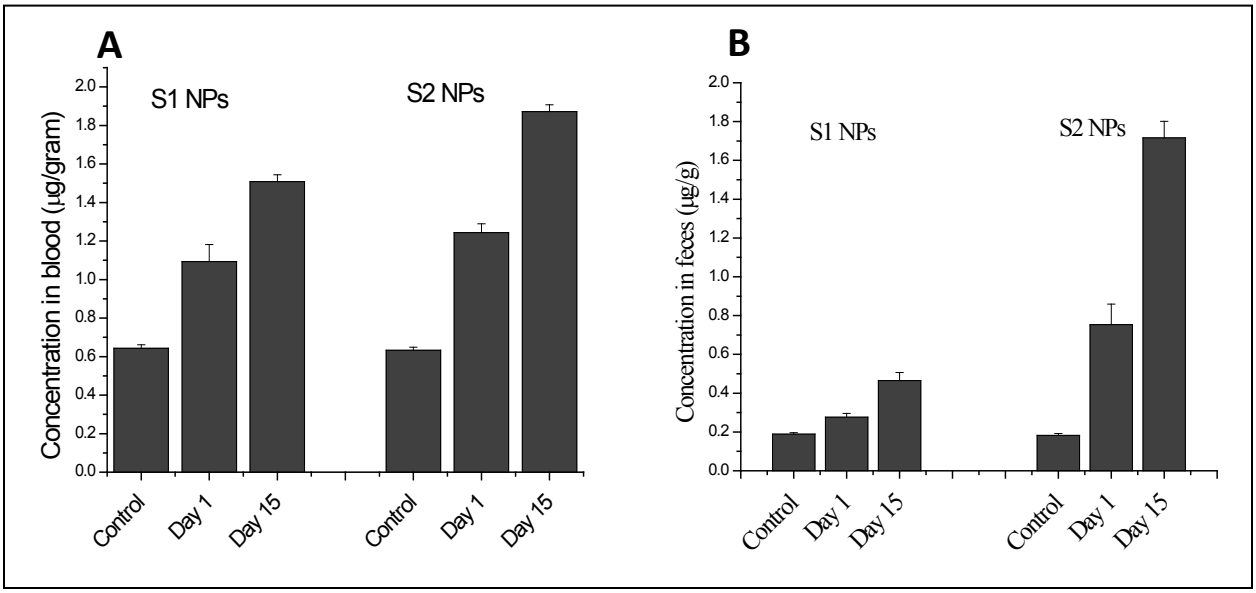


**Figure 4.14:** S1NPs or S2NPs levels in blood and feces as indicators of inorganic NPs circulation and elemination at 1000 µg/mL dose. **(A)** Exhibit concentration of S1NPs and S2NPs in blood. **(B)** Concentration of S1 and S2 NPs in feces. Day-0 to day 15 is the time interval during which the NPs were administered. n = 6. Values are expressed as mean±SEM; *superscripts indicate significant differences ($P<0.05$) compared with the control group.

The kinetics of S1NPs and/or S2NPs in the blood and feces was determined by measuring the S1NPs or S2NPs concentrations in successively collected samples at highest dose of 1000µg/Kg body weight. Fig. 4.14A displayed 1.13 folds and 1.34 folds higher concentrations of S2NPs in blood compared to S2NPs at both time points. As shown in Fig. 4.14B, the elimination rate of S2NPs in the feces of mice were 2.77 folds and 4.25 folds higher compared to S1NPs.

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### 4.4.2.4. Cytokines and Apoptotic markers analysis:

Pro and anti-inflammatory cytokines levels were estimated compared to the control from serum of Balb/C mice after the treatment with S1NPs and S2NPs for 15 days for dosages of 100-1000µg/Kg body weight. As shown in Fig. 4.15, TNF-α level of S1NPs increased 1.87, 2.48, 3.59, 5.37 folds significantly (Fig. 4.15) and IL-10 level decreased by 1.21, 1.71, 2.13 and 3.77 folds significantly. S2NPs increased the TNF-α level by 1.48, 1.87, 2.58, 3.09 folds but depleted IL-10 level by 1.03, 1.21, 1.50, 1.86 folds.

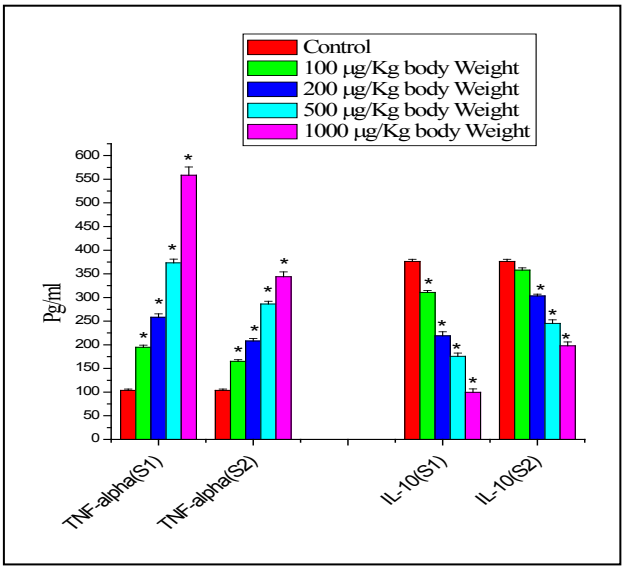


**Figure 4.15:** Cytokines analysis *in vivo*. Analysis of Pro and anti-inflammatory cytokine level of S1NPs and S2NPs treated mice serum after 15 days (TNF-α, IL-10). Values are expressed as mean±SEM; n=6; *superscripts indicate significant differences (P<0.05) compared with the control group.

The pro and anti-apoptotic protein expression levels after 15 days treatment were enumerated in Table 4.4, compared to control group. As shown in Fig. 4.16A, both S1NPs and S2NPs displayed

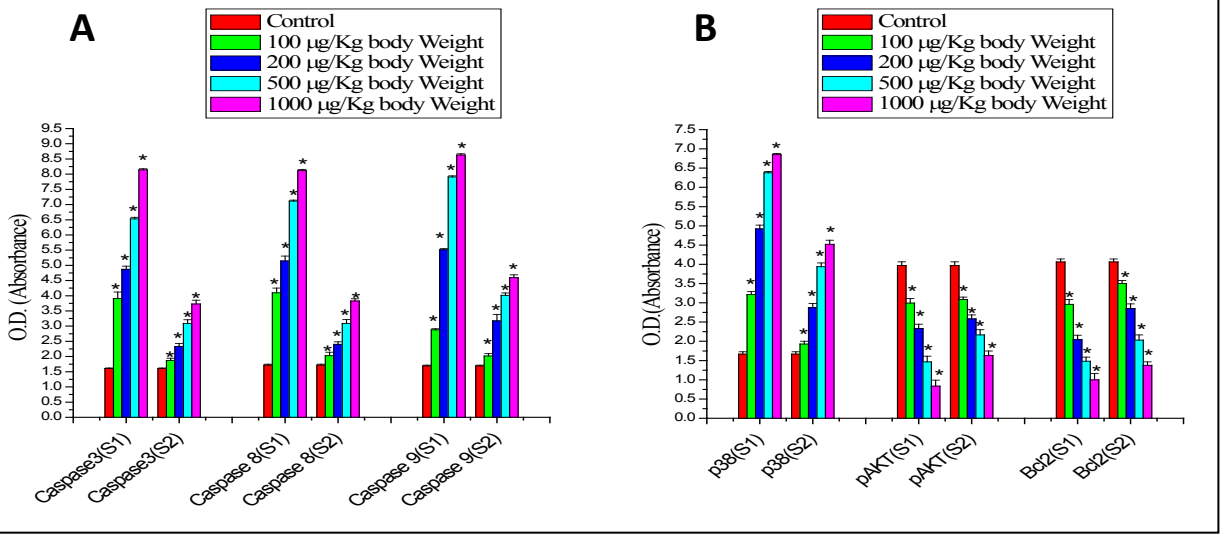


**Figure 4.16: (A,B)** Assessment of pro-apoptotic (Caspase-8, Caspase-9, Caspase-3, p38) and anti-apoptotic (pAKT, Bcl2) protein level by ELISA after S1NPs and S2NPs treatment. Values are expressed as mean±SEM; n=6; *superscripts indicate significant differences (P<0.05) compared with the control group.

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likewise increasing trends of the pro-apoptotic protein (Caspases-3, 8, 9) expression level with increase in doses compared to the control. However, the anti-apoptotic expression level of S1NPs and S2NPs (Fig. 4.16B) down-regulated in a similar manner for increase in doses. Of note, the up-regulation and down-regulation of S1NPs were higher compared to the S2NPs.

| Apoptotic Markers | 15 Days Different Doses (µg/Kg body weight) | | | |
|---|---|---|---|---|
| | 100 | 200 | 500 | 1000 |
| Caspase 3 (S1) | 2.43 | 3.04 | 4.07 | 5.08 |
| Caspase 8 (S1) | 2.39 | 3.00 | 4.15 | 4.74 |
| Caspase 9 (S1) | 1.70 | 3.26 | 4.68 | 5.10 |
| P38(S1) | 1.93 | 2.96 | 3.84 | 4.12 |
| pAKT(S1) | 1.32 | 1.69 | 2.71 | 4.77 |
| Bcl2(S1) | 1.4 | 1.99 | 2.74 | 4.06 |
| Caspase 3(S2) | 1.23 | 1.53 | 2.02 | 2.45 |
| Caspase 8(S2) | 1.38 | 1.62 | 2.09 | 2.59 |
| Caspase 9 (S2) | 1.27 | 2.00 | 2.53 | 2.90 |
| P38(S2) | 1.14 | 1.70 | 2.33 | 2.69 |
| pAKT(S2) | 1.16 | 1.39 | 1.71 | 2.25 |
| Bcl2(S2) | 1.12 | 1.37 | 1.94 | 2.86 |

**Table 4.4:** Representation of changes in pro and anti-apoptotic markers from mice serum after treatment with S1NPs and S2NPs at different doses (100, 200,500 1000µg/Kg body weight) for 15 days. Values were expressed as a change in fold compared to control.

#### 4.4.2.5. Histopathological Study:

After 15 days, at 100µg/ Kg bodyweight S1NPs treated Balb/C mice exhibited minor changes in hepatocytes arrangement. Whereas for 200-1000µg/Kg bodyweight, disorganization of the hepatocytes, hemocyte overfilling in blood vessels, hepatocyte enlargement, focal lymphocytic infiltration, focal necrosis, diffused vacuolated hepatocytes, loosened liver parenchyma, disarrangement of hepatic lobules, central vein dilation and disruption were observed (Fig. 4.17). Whereas S2NPs treated mice showed disorganization of the hepatocytes and loosened liver parenchyma but only at higher doses (Fig. 4.17) (500-1000µg/Kg bodyweight).

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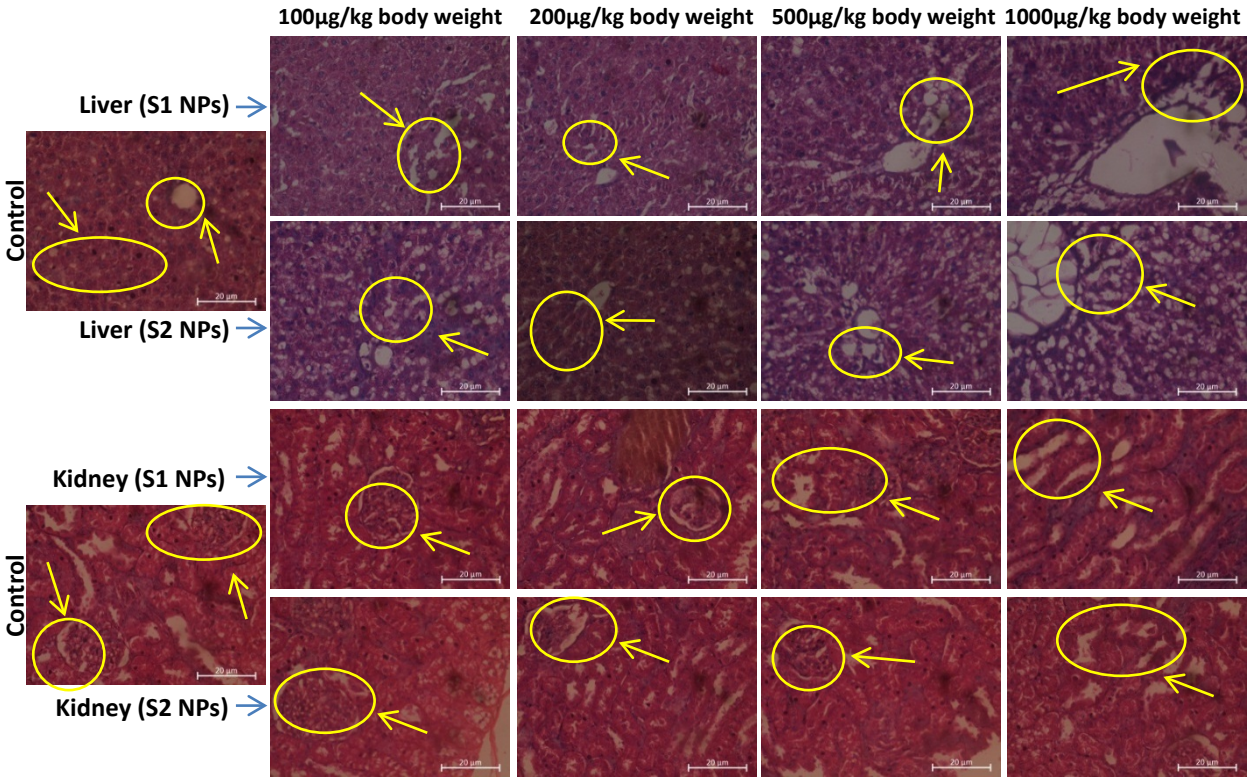


**Figure 4.17:** Histological images of Liver and Kidney. Histological images of hematoxylin-eosin stained Liver and Kidney of Balb/c mice after treatment with different doses (100-1000µg/mL) of S1NPs and S2NPs after 15 days. The circles in the microscopic image of Liver indicate the areas of leison in liver tissue compared to the control Liver tissue and in case of kidneys; the circles indicate histopathological areas of changes due to toxicity compared to the control tissue.

In kidney of S1NPs treated mice, swelling and dilation of Bowman's capsule, deposition of hyaline-like materials in proximal tubules, degeneration changes in epithelium of the proximal tubules as well as rupture of Malpighian corpuscles and Bowman's capsule (Fig. 4.17) were observed for 100-1000µg/Kg bodyweight. However in kidney of S2NPs treated experimental group, no significant toxicity was observed for 100-200µg/Kg bodyweight. Furthermore, in kidney of S2NPs treated experimental group, comparatively lesser swelling of Bowman's capsule and deposition of hyaline-like materials in only some proximal tubules were observed (Fig. 4.17) for 500-1000µg/Kg bodyweight. Lastly, disarrangement of proximal tube and rupture of Bowman's capsule were prominently observed at 1000µg/Kg bodyweight in S2NPs.

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## 4.5. DISCUSSION:

In the *in vitro* study, it was aimed to distinguish the biodistribution and toxicity differences between S1NPs and S2NPs by investigating the Cu ions internalization inside the lymphocytes, biochemical estimation, ROS generation, apoptotic study, pro and anti-apoptotic levels and cytokines estimation. The intracellular concentration of S1NPs was considerably higher compared to S2NPs after administration with 100µg/mL doses for 24h in lymphocytes (Fig. 4.2). S1NPs and S2NPs toxicity were estimated using human lymphocytes as measured by the MTT and several enzymatic markers. The proportion of viable cells declined after S1NPs or S2NPs exposure compared to control. It was observed that % of cell death by S1NPs was higher than S2NPs at all doses, indicating greater cell viability of S2NPs. The higher % of cell death of S1NPs may be attributed to higher intracellular concentration of S1NPs compared to S2NPs. The upstream of various enzymatic (LDH, NADPH and MDA) and non-enzymatic processes implied adverse effects on the cell membrane integrity (Burd and Usategui-Gomez, 1973), signal transduction, enzymatic reactions, mitochondrial electron transport chain and gene expression, DNA (Zhuang and Simon, 2000; Stohs and Bagchi, 1995) through the generation of ROS (Frädrich et al., 2016). The upstream of enzymatic markers after NPs (S1NPs and S2NPs) exposure suggested significant ROS generation.

Fig. 4.2 showed that both S1NPs and S2NPs significantly induced the intracellular production of ROS in human lymphocytes. Overproduction of ROS has been shown to play an important role in oxidative stress through oxidative DNA and protein damage (Droge W, 2002). It was observed that S1NPs displayed higher ROS generation levels compared to S2NPs, indicating greater toxicity of S1NPs.

The results presented in Fig. 4.6 provided strong evidence that both S1NPs and S2NPs induced apoptosis in lymphocytes with the production of ROS acting as a signaling molecule for the initiation and execution of the apoptotic cell death mechanism (Ott et al., 2007). It can be emphasized that S1NPs treated lymphocytes displayed more apoptosis compared to S2NPs. The apoptotic/necrotic study also revealed that S1NPs displayed higher degradation of lymphocytes shape and late apoptosis compared to S2NPs (Fig. 4.6C-D). Previous study demonstrated that, oxidative stress plays a vital role in apoptosis induced by CuONPs in human lung epithelial (A549) cells (Fahmy and Cormier, 2009).

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In the *in vivo* study, it was aimed to distinguish the biodistribution and toxicity differences between S1NPs and S2NPs by examining the body weight, organ weight, organ distribution, biochemistry, histology, pro and anti-apoptotic levels and cytokines estimation. First, reduction of body weight was observed in S1NPs treated mice after intraperitoneal administration for 100-1000µg/Kg bodyweights after 15 days. Whereas, bodyweight decreased in S2NPs experimental groups only at higher doses (500-1000µg/Kg bodyweight). Mass increase in liver and kidney but reduction in spleen weight at all respective dosages was observed. The intraperitoneal exposure of inorganic NPs has been shown to influence the body and organ weight (Zhang et al., 2010). However, changes in liver, kidney and spleen mass by S1NPs were significantly higher than that of S2NPs. Further, heart mass showed no significant changes in both experimental groups.

At 1000µg/Kg bodyweight after 15 days, the majority of the S1NPs and S2NPs were accumulated in the liver and spleen. The liver and spleen being part of the mononuclear phagocyte system (MPS), the accumulation could be attributed to the uptake by the phagocytic Kupffer cells in the liver and the macrophages and B cells in the spleen (Hirn et al., 2011; Xue et al., 2012; Sonavane et al., 2008). A relatively large amount of S1NPs accumulated in the kidney, lungs, heart and intestines.

Choi et al. (2007) demonstrated that NPs with sizes <5.5nm manifested rapid urinary excretion. Hence renal excretion of S1NPs and S2NPs with sizes of 11.25±3nm and 30.6±8nm respectively could not be the primary route of elimination. Furthermore, significantly higher S2NPs and very few S1NPs were detected in the feces, indicating that the S2NPs but not the S1NPs were excreted through the biliary pathway from the liver to the bile duct, intestine and feces (Khlebtsov and Dykman, 2011).

High levels of TNF-α, induced by NPs act as a regulator of acute inflammation (Fishman and Perelson, 1999). In this study, the significant dose-dependent up-regulation of pro-inflammatory cytokine TNF-α and down-regulation of anti-inflammatory cytokine IL-10 indicated that both S1NPs and S2NPs increased the inflammatory response, with S1NPs inducing higher inflammatory response *in vitro* and *in vivo*. Similar result was demonstrated by Chattopadhyay et al. (2015).

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TNF-α triggers apoptosis through activation of initiator caspase, Caspase-8. The Caspase-8 along with the Caspase-9 and 3 mediates mitochondria-operated pathways of apoptosis (Kuang et al., 2016; Xu et al., 2012). In the present study, significant up-regulation in the activities of Caspases-3, 8 and 9 and mitogen-activated protein kinase (MAPK) p38 and down-regulation of pAkt and Bcl-2 was observed after S1NPs or S2NPs exposure, indicating that S1NPs and S2NPs induced caspase mediated mitochondrial targeted apoptotic signaling pathways *in vitro* and *in vivo* (Ivanov and Ronai, 2000; Pan et al., 2010). The study also found that Caspases were more sensitive to S1NPs than S2NPs *in vitro* and *in vivo*.

From the histopathological study, it was observed significant toxicity in case of S1NPs. After the 15 days treatment with S1NPs, it was observed adverse toxicological impact on liver and kidney but S2NPs treated mice showed comparatively less toxicological impact on liver and kidney tissues. Toxicological effects of CuNPs have been shown in kidney, liver and spleen (Chen et al., 2006) which is in agreement with this present study. Also the dysfunction of liver and kidney as indicated by higher SGOT and creatinine levels of S1NPs and S2NPs reflects the histopathological study.

The NPs morphology, surface functionalization and ion dissolution were major influences in biodistribution and toxicity results of NPs by aiding cellular uptake and translocation of the NPs *in vitro* and *in vivo* (Nel et al., 2006; Kang et al., 2013). The present study elucidates that, rectangular shaped S1NPs with 11.25±3nm diameter significantly killed more normal lymphocytes than spherical shaped S2NPs of 30.6±8nm diameters for doses ranging from 1-100µg/mL. The lower toxicity of S2NPs might be attributed to sustained release of S2NPs due to its shape and surface capping agents. Abe et al. (2012) demonstrated that the surface modification of CuONPs reduced its toxicity outcomes compared to uncoated CuONPs by precluding intimate contact between cells and NPs. The dissolution of NPs and exertion of toxic ions proved to be susceptible to $p^H$, composition and exposure time (Xia et al., 2008). Previous studies manifested that the surface coating of NPs controlled the composition and structure of the complex protein corona which was formed immediately when NPs were incubated in a biological fluid which was similar to the pathophysiology *in vivo* (Tenzer et al., 2013; Jedlovszky-Hajdú et al., 2012; Sakulkhu et al., 2014). NPs were stored primarily in the liver and spleen, which then released ions that migrated and accumulated in several major organs (Su et

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al., 2014). Cho et al. (2011) demonstrated that dissolution of metal oxide NPs inside of phagosomes was the main cause of NPs induced toxicity. S1NPs with higher dissolution rates might be responsible for greater toxicity compared to S2NPs particles.

The present study indicates that green CuONPs is less toxic compared to Chemical CuONPs. Green CuONPs may be used as a drug delivery system and in several biomedical applications in cancer. However further studies involving the beneficial properties of green CuONPs compared to chemical CuONPs are needed to gather valuable information for safety.

It was demonstrated the *in vitro* and *in vivo* study of S1NPs and S2NPs after their synthesis and physical characterization. The *in vitro* study demonstrated that S1NPs showed more Cu ions internalization and hemolysis than S2NPs in lymphocytes after 24h. The findings of biochemical markers, ROS generation and apoptosis clearly indicate greater toxicity of S1NPs compared to S2NPs *in vitro*. The *in vivo* study demonstrated that the S2NPs were mainly stored in the liver and spleen, whereas the S1NPs were widely stored in more organs including the heart, lungs, kidney and intestine. The NPs circulation in the blood and excretions in the feces were also found to differ between the S1NPs and the S2NPs. Definite signs of toxicity in mice treated with S1NPs and/or S2NPs were observed over the period of 15 days as indicated by observing the body and organ weight, organ distribution, biochemistry and histology, with S1NPs suggesting greater toxicity than S2NPs. The levels of biomarkers of oxidative stress, apoptosis and cytokines were significantly altered in lymphocytes (after 24hr) and mice serum (after 15 days) treated with both NPs, while more effective change was observed in S1NPs group. These findings suggested that NPs physiochemical compositions (S1NPs and S2NPs) were individually responsible for their distribution and toxicity outcomes *in vitro* and *in vivo*.

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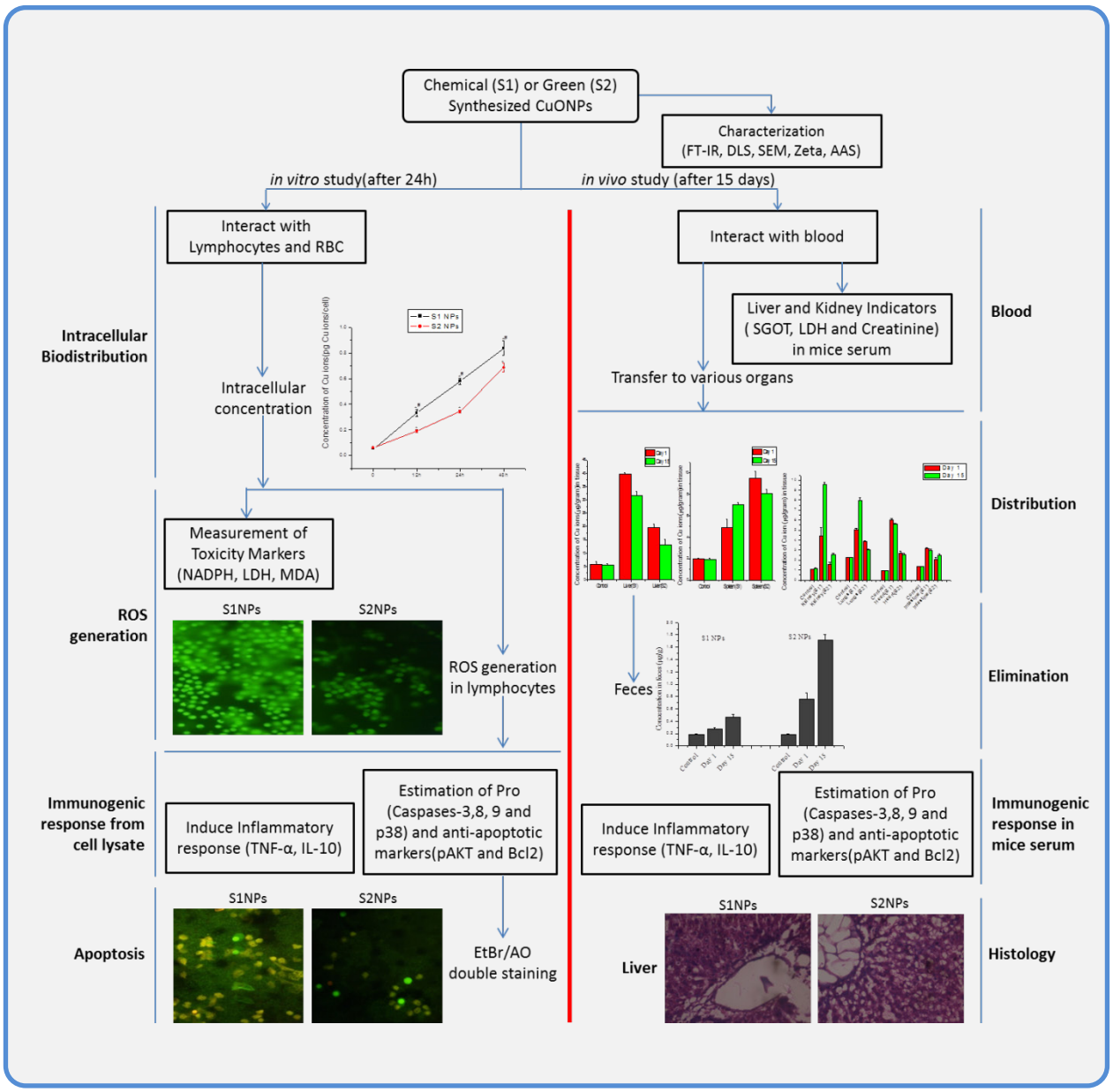


Comparative study of toxicity parameters between chemical (S1) and green (S2) synthesized CuONPs

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