5. BIOCOMPATIBILITY, TOXICITY PROFILING AND DEGRADATION OF NANOPARTICLES

Nanotechnology is a multidisciplinary field involving principles from chemistry, biology, physics, and engineering to design and formulate nanoscale devices.[1-3] The field of medicine stands to be a significant benefactor of advances in nanotechnology due to improvement in detection, imaging, and therapy of disease.[4-7] Nanomedicine is a vast subject area which covers variety of nanoparticles, nanomachines, nanofibers and polymeric nanoconstructs as biomaterials, and nanoscale microfabrication-based devices, sensors and laboratory diagnostics. Currently, research into the rational delivery and targeting of pharmaceutical, therapeutic, and diagnostic agents via intravenous and interstitial routes of administration by means of nanosized particles is at the forefront of projects in nanomedicine.[8]

The discipline that studies structural and functional cellular derangements produced by particles of 1–100 nm in size is termed as "Nanotoxicology".[9,10] Many of the toxic properties exhibited by nanomaterials are associated with ultrastructural alterations within the cell.[11] These changes are dependent on the dose, size, shape, surface area, aggregation, and surface functional conjugation of the materials.

Nanoparticles to be used in a drug delivery system should be nontoxic in vivo.[12] Interestingly, a wide variety of other nanostructured and microstructured materials have been shown to be biocompatible with various cells and tissues.[13,14] Many of them have also been investigated for biological and medical applications, such as drug delivery[15-21] and bioimaging.[11] A few types of nanoparticles have shown good promise and have been commercialized as smart delivery vehicles or diagnostic agents for various diseases.

Recent breakthroughs on the architectural control and surface functionalization of inorganic nanomaterial-based delivery vehicles, such as mesoporous silica nanoparticles (MSNs),[22-25] have brought new possibilities to this burgeoning area of research. Among inorganic nanomaterials, mesoporous silica nanoparticles are centre of focus because of their unique properties.[26] Since the first report of use of mesoporous silica nanoparticles (MSNs) as drug delivery system in 2001[26,27], an exponential raise in research on biomedical application of MSNs has been observed in the last few years. The continuously increasing requirements from clinical patients for high-

performance therapeutic nanoformulations are the main reason for the worldwide research interest in biomedical applications of MSNs. MSNs as nanometer-sized drug carriers are expected to overcome some of the problems of current nanoformulations[26] as MSNs possess numerous attractive features which include large surface areas, tailorable pore sizes, controllable particle sizes and shapes, and dual-functional surfaces (exterior and interior).[28] Owing to uniquely ordered nanoporous structure and high surface area as well as large pore volume, MSNs could achieve a higher loading of active ingredients and a tunable drug release profile due to which they exhibit excellent performance in both controlled drug delivery and formulation of poorly soluble drugs to enhance bioavailability.[29]

As nanocarriers, MSNs have been explored as effective drug delivery systems for a variety of therapeutic agents to fight against various kinds of diseases including bone/tendon tissue engineering,[30-32] diabetes,[33] inflammation,[34] and cancer.[35-42] Conjugation of MSNs with definite ligands such as folic acid[43], lectins[44] etc, or antibodies allows targeting of nanoparticles to special cell types like cancer. Furthermore, modification of MSNs surface with variety of stimuli responsive nanovalves or polymer capping confer specific controllable capability over drug release so that any premature release can be prevented and maximum drug can be delivered to target site which opens unlimited possibilities of various controllable delivery systems.[45-59]

Concerns about the potential toxicity of MSNs in human health and on the environment have also been raised[60-65] with intensive research efforts toward the syntheses of various MSNs and their potential applications in biology and medicine[66-72]. Though MSNs are a promising material for drug delivery and research on biomedical application of MSNs is increasing day by day, considerable research and investigation needs to be done and number of questions remain to be answered before MSNs can be used as a drug-delivery system. Very few data regarding the in vivo biocompatibility of MSNs is available. Studies on the toxicology of MSNs is also very limited.[73] Though the number of studies regarding the safety and toxicity of MSNs has increased rapidly during the last years[74], quite conflicting results have been documented which makes the biocompatibility of MSNs a subject of intense debate.[75-82] These conflicting results may arise from differences in composition, size, structure, surface areas, surface charges, residual reagents and templates, and route of administration of the mesoporous materials as well as the type of cells or tissues studied.[83] In addition to this, slight variations in the existing synthetic procedures

and postsynthetic modifications of MSNs can result in an array of MSNs with different structural and surface properties. On the other hand, the complexity of biological conditions under which the nanoparticles are applied can also lead to a range of unpredictable biological responses to MSNs.[84]

Some recent studies reported that no severe toxicity to mice was observed when used in the short term at a concentration required for in vivo imaging. Hudson et al. reported that intraperitoneal or intravenous administration of 1.2 g kg^{-1} MSNs is lethal to SV129 mice but is safe when reduced to 40 $mg.kg^{-1}$.[85] In 2014, Roggers et al. reviewed the literature on the use of MSNs for systemic drug delivery in vivo; although they concluded that the MSN was a promising candidate for a drug delivery vehicle, they also stated that to fully understand its toxicity, biodistribution and excretion, methodological consensus and systematic in vivo studies are needed.[86]

As per our knowledge all the research regarding biocompatibility and toxicity of MSNs conducted till date utilized alkoxide compounds as a silica source. Recently, the use of a silicate precursor as a substitute for alkoxide compounds has increased in modern materials science, because of the fact that these materials are economic and non-toxic. Hence, silicate precursors are preferable to alkoxide compounds in the framework of environmentally friendly processes.[87]

Furthermore, questions regarding the metabolism and clearance of MSNs from biological systems are raised[88,89] as they are generally recognized to be more stable than organic drug delivery carriers[90]. Moreover, the accumulation of MSNs in tissue can also cause toxicity, which can be avoided by biological degradation.[91] Hence, the study regarding the degradation of MSNs is also very crucial.

In the present study, MCM-41 type of MSNs were synthesized using sodium silicate as an economic source of silica. The synthesis involved many variations in synthetic procedures as compared to those used for synthesis of MSNs using alkoxides. This change in the silica source can lead to difference in variety of physicochemical parameters such as composition, size, shape, porosity etc. which are significant contributors to the biocompatibility of MSNs. Furthermore, the incorporation of CuO (in case of CuO-MSNs) may also show some toxicities. Hence, it was

necessary to confirm that the synthesized MSNs were nontoxic in nature and biodegradable before going ahead with these MSNs as a nanocarrier for drug delivery.

5.1 Hemolysis study:

For hemolysis assay, the red blood cells (RBCs) from chicken were obtained from government approved slaughter house. The fresh blood was collected in EDTA treated tubes plasma was removed as supernatant by centrifugation at 3000 rpm for 10 min, refined by successive rinsing with PBS buffer (pH 7.4). The suspension of RBC was diluted 10 times with PBS buffer (pH 7.4), and then 200 µL of RBCs suspension was added to 800 µL of MSNs or CuO-MSNs with different concentrations (1 - 200 µg/mL). In the case of positive control, 200 µL of RBCs suspension was added to 800 µL Triton X100 (2% v/v), and for negative control 200 µL of RBCs suspension was added to 800 µL of PBS buffer (pH 7.4). Afterwards, all of the samples were incubated for 4 h in shaker incubator instrument. Finally, the samples were centrifuged at 10,000 rpm for 2 min, and the absorbance of supernatant (hemoglobin) was measured by UV-visible spectrophotometer at 541 nm. The hemolytic activity percentages of the different samples were calculated as follows:[92]

$$\%\ \text{Hemolysis} = \frac{\text{Abs(sample)} - \text{Abs(ctrl}-)}{\text{Abs(ctrl}+) - \text{Abs(ctrl}-)} * 100 \qquad (1)$$

5.2 In vivo toxicity studies:

Animals have been used as sentinel for early detection of potential risk to humans. Toxicity testing in animals is conducted to identify possible adverse effects resulting from exposure to an agent or to develop dose response relationships that allow evaluation of responses at other exposures.[93]

The single dose as well as multiple dose toxicity study of synthesized MSNs and CuO-MSNs was carried out in three consecutive steps: Acute toxicity, sub-acute toxicity and chronic toxicity.

5.2.1 Acute toxicity study:

Acute toxicity tests evaluate the adverse effect of short term exposure and are considered by EPA (Environmental Protection Agency) to be an "Integral step in assessment of toxic potential under the regulatory framework of its pesticide and toxic substances programs" (EPA 1998a). To be considered an acute exposure, dosing may be done once or may be done several times within or continuously throughout 24h time period but use of a single dose is a far more common method.[93]

Acute toxicity study involved examination of toxicity of MSNs and CuO-MSNs at given doses after single I.V. injection. For acute toxicity profiling, 27 female CD-1 mice were selected and divide into nine groups (control group, I-VIII test group; each containing 3 mice). Each mouse of control group was administered with saline. Each mouse of test group I-IV was injected through the tail vein with MSNs dispersed in 0.1ml saline solution with a dose of 5mg/kg, 10 mg/kg, 20mg/kg and 40mg/kg of mice (n=3 for each dose) while every mice of V-VIII test group, were injected through the tail vein with CuO-MSNs dispersed in 0.1ml saline solution with a dose of 5mg/kg, 10 mg/kg, 20mg/kg and 40mg/kg of mice (n=3 for each dose). Following parameters were monitored every day throughout study.

- Body-weight change,
- Visible and/or palpable dermal infection,
- Presence of ascites,
- Grooming or impaired mobility.

A Body-condition scoring system was also used to evaluate the nutrition status of the mice.[94] After 14 days, approximately 100 µl of the blood was collected from retroorbital plexus into a tube containing dipotassium EDTA and characterized for hematological parameters (complete blood count; CBC).

5.2.2 Sub-acute toxicity study:

Sub-acute studies evaluate the adverse effects of continuous or repeated exposure over a portion of the average life span of experimental animals.[93]

Sub-acute toxicity study involved measurement of toxicity of MSNs at given doses after multiple injections for short time duration. 27 female CD-1 mice were selected and randomly allocated into nine groups (control group and I-VIII test group, each containing 3 mice). Each mouse of control group was administered with saline. Each mouse of test group I-IV was injected through the tail vein with MSNs dispersed in 0.1ml saline solution while each mouse of V-VIII test group, was injected through the tail vein with CuO-MSNs dispersed in 0.1ml saline solution with a dose of 5mg/kg, 10 mg/kg, 20mg/kg and 40mg/kg of mouse (n=3 for each dose), twice weekly up to 14 days (total 5 injections). All the parameters monitored during acute toxicity studies were monitored daily and after 14 days, CBC analysis was performed.

For testing of biochemical parameters, the blood, collected into a tube, was allowed to clot and the serum was separated by centrifugation. Variety of biochemical parameters such as:

- Glucose, creatinine, blood urea nitrogen (BUN), AST, ALT, GGT, total protein, total bilirubin, albumin etc. were examined.

Animals were sacrificed by over dose of thiopentone sodium and tissue portions from the seven different organs such as brain, spleen, liver, heart, lungs, intestine and kidney were collected for sectioning and stored in 10% formalin for histopathological examination. These formalin-fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin stain (H&E) for histopathological examination under a light microscope.

5.2.3 Chronic Toxicity profiling:

The purpose of chronic toxicity testing is to determine the cumulative adverse effects of repeated exposures of test animals to various doses of a material.

Maximum tolerated dose observed during acute and sub-acute toxicity study was selected for chronic toxicity profiling. CD-1 female mice were randomly allocated into three groups, control group and test group I, II (each containing 3 mice). All the test group I mice were injected through the tail vein with 40mg/kg of MSNs dispersed in 0.1ml saline solution while test II group mice were injected through the tail vein with 40mg/kg of CuO-

MSNs dispersed in 0.1ml saline solution twice weekly up to 60 days. Various parameters evaluated during the studies and at the end of studies, were similar to those performed during sub acute toxicity studies.

5.3 Degradation of MSNs:

5.3.1 In vitro degradation of MSNs:

The in vitro degradation of as synthesized MSNs and CuO-MSNs was studied in phosphate buffer (pH 7.4) using an activated dialysis bag. 5mg of MSNs and CuO-MSNs were dispersed seperately in media by sonication for 2 minutes, seized in dialysis membrane and it was placed in a beaker containing 100 mL of diffusion media with 100 RPM magnetic stirring. Samples (1 mL) were withdrawn periodically and replaced with the same volume of fresh medium at regular time interval of every day up to 6 days. The amount of silica solubilized in medium as silicic acid (degradation product of silica) was measured by performing previously described procedure based on molybdenum blue with little modifications.

The aliquots collected at specified time intervals were treated with 0.1ml of 7.5M sulfuric acid followed by reaction with 10% w/v ammonium molybdate solution. The resulting solution was diluted with DI water upto 4ml and kept aside for about standing to allow complete formation of yellow molybdosilicic acid. About 2 ml, after centrifugation and filtration, was acidified by addition of 1ml 7.5 M sulfuric acid solution and 0.9ml 10% tartaric acid, 0.1ml 0.1% potassium antimonyl tartarate solution. At the end 1ml 1% ascorbic acid solution was added and solution was kept aside for about 15 min. Absorbance of resulting solution was measured at 810 nm against a reagent blank prepared in the same way, 15 min after mixing the solutions. Calibration curve was prepared in similar manner with known amounts of silica from the standard solution of sodium silicate.

5.3.2 In vivo degradation of MSNs:

To determine the in vivo degradation of synthesized MSNs, we injected three female wistar rats with MSNs (20 mg/kg). The urine and feces collected by special metabolic cages for rats were analyzed with spectrophotometric method based on molybdenum blue. The urine and feces of control group rats (no injection) were treated with reagents in a similar manner

and used as a blank. Calibration curve was prepared in similar manner with known amounts of silica using the standard solution of sodium silicate in urine.

5.4 Results and Discussion:

5.4.1 Hemolysis study:

Hemocompatibility of nanoparticles can be confirmed by in vitro assay to evaluate biosafety of synthesized nanoparticles on erythrocytes. The percent hemolysis of RBCs was calculated for different concentrations of MSNs as well as CuO-MSNs. As illustrated in Figure 5.1, both MSNs and CuO-MSNs exhibited excellent biocompatibility (MSN: 1.87% hemolysis, CuO-MSNs: 2.48% hemolysis) even at concentration as high as 200 µg/ml.

Though the percent hemolysis exhibited by CuO-MSNs was very less even at higher concentration, it was little higher than the hemolysis showed by plain MSNs. This might be due to presence of CuO incorporated within CuO-MSNs framework which may cause hemolysis.

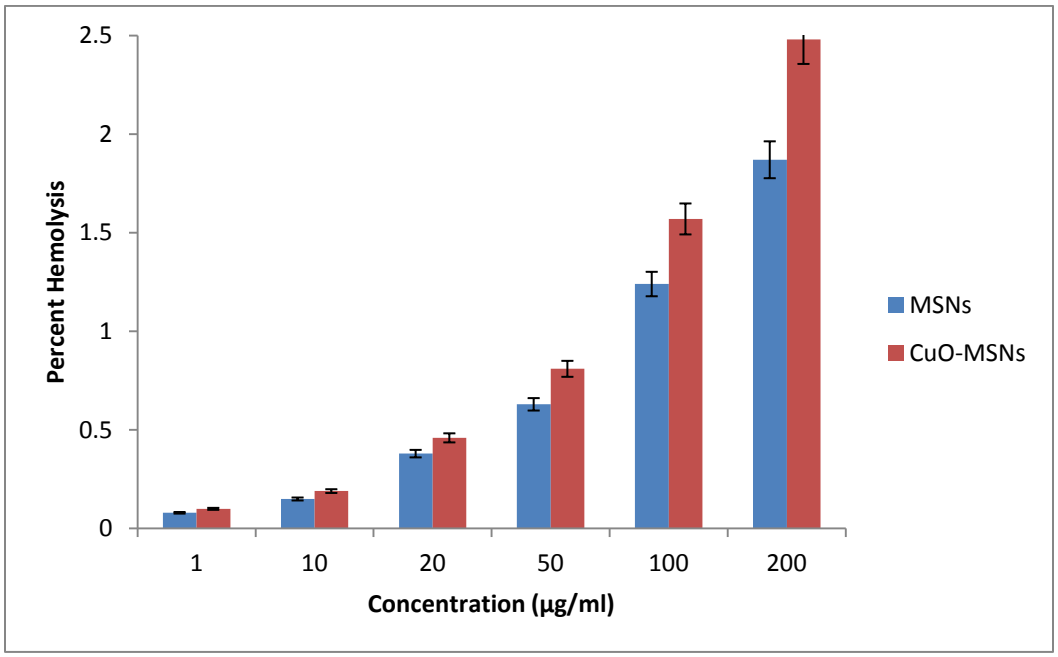


Figure 5.1: Hemolytic activity of MSNs against chicken blood cells.

5.4.2 In vivo toxicity studies:

5.4.2.1 Acute toxicity:

Acute toxicity data have benefits beyond toxicity ranking as acute studies reveal whether toxicity is sudden, delayed, time-limited or continuous.

The body weights of all treated mice were compared with the body weight of control mice and no significant difference was observed, indicating no effect on body weight at even the highest dose (40 mg kg^{-1}), as shown in Figure 5.2 (represent mice treated with MSNs) and 5.3 (represent mice treated with CuO-MSNs). No signs of any dermal change, impaired mobility, or reduced food intake were observed in any trestment group. The body condition scores (BCS) of all mice were 3, confirming that all mice were well conditioned according to the published scale.[94] Dipotassium EDTA causes less post collection platelet clumping and provides better staining characteristics and hence it was preferred over heparin as an anticoagulant for rodent blood.[95]

Table 5.1 and 5.2 provides the information regarding the hematological parameters determined by using the auto hematology analyzer BC-2800Vet (Mindray, China). All mice including control group were found to possess platelet count less than the normal range. Though the platelet count of treatment groups was lesser than the normal range, it was comparable to the control group which also showed low platelet count and hence, it can be concluded that the reduced platelet count wasn't due to MSNs or CuO-MSNs and nanoparticles did not show any significant toxicity in mice after 40 mg/kg single dose. Two mice treated with 5 mg/kg and 10mg/kg of CuO-MSNs, showed little decrease in %RDW (12.3 and 14.4, respectively; Normal range: 14.7-19.1%). Low % RDW, just indicates a uniformity in the size of red blood cells and hence, it is not of health concern.

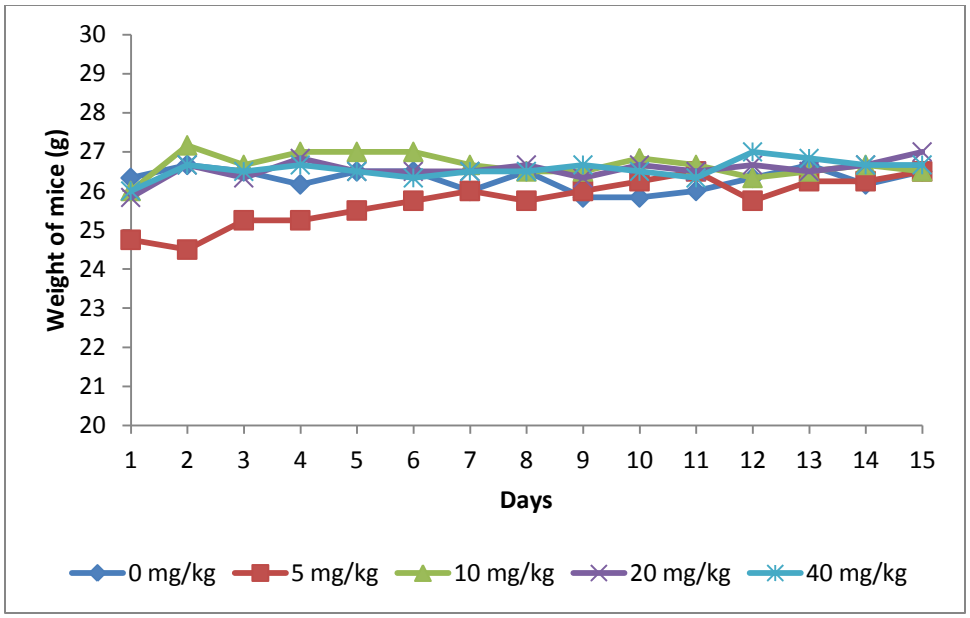


Figure 5.2: Average body weight of mice treated with single dose of MSNs (Acute toxicity) .

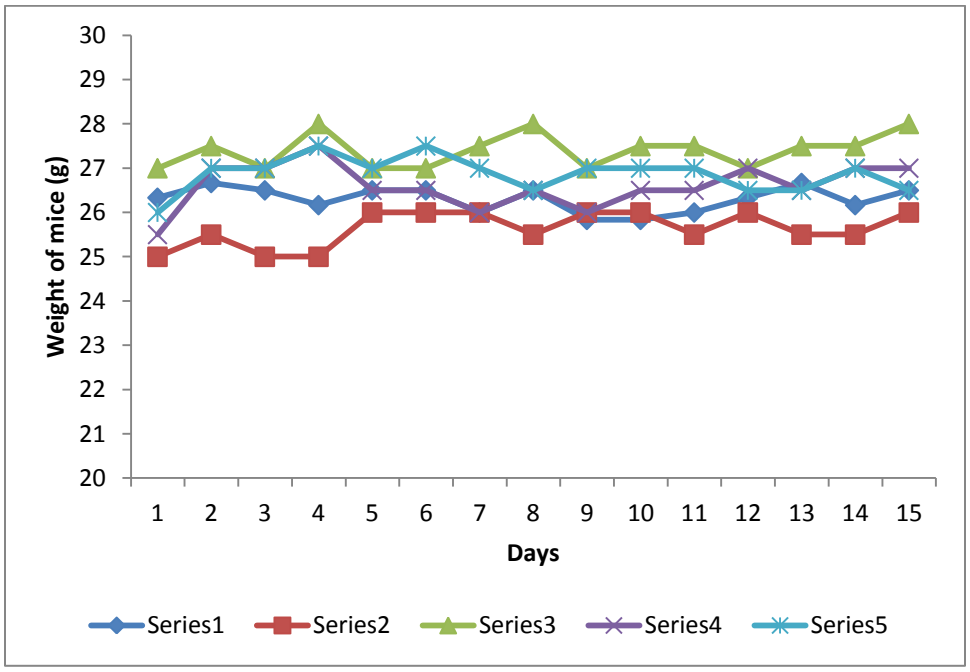


Figure 5.3: Average body weight of mice treated with single dose of CuO-MSNs (Acute toxicity) .

Table 5.1: Hematological parameters of mice given single dose of MSNs (Acute toxicity).

Parameters	Reference Range	Control	Dose of MSNs (mg/kg/mice)			
		0	5	10	20	40
WBC * 10^3	4.58-16.21	5.47±1.41	5.17±1.07	6.67±0.50	5.33±0.90	6.83±0.55
Lymph* 10^3/µl	2.68-11.64	3.93±0.96	3.67±1.02	4.67±0.55	4.07±0.61	4.73±0.57
Mon* 10^3/µl	0-1.49	0.17±0.06	0.2±0.1	0.37±0.06	0.07±0.06	0.2±0.1
Gran* 10^3/µl	0.55-4.53	1.37±0.64	1.33±0.49	1.63±0.11	1.2±0.36	1.9±0.3
Lymph%	43.35-86.46	72.16±5.8	70.55±7.87	69.85±3.27	76.41±1.94	69.14±3.68
Mon%	0-13.44	3.35±1.72	3.71±1.21	5.56±1.22	1.37±1.21	2.95±1.44
Gran%	7.48-50.04	24.5±6.51	26.36±9.44	24.59±2.49	22.21±3.11	27.91±4.66
RBC* 10^6	7.17-11.35	7.37±0.15	7.63±0.21	8.07±0.49	7.3±0.35	8.07±0.51
HGB g/dl	11.2-	13.2±0.89	13.6±0.66	12.7±0.83	11.7±0.81	12.5±0.96

	17.8					
HCT%	38.2-64	42.77±2.95	43.67±1.83	49.4±0.44	43.1±2.55	42.33±2.17
MCV fl	47.5-66.7	48.03±0.67	48.1±0.53	57.1±0.26	49.8±0.24	50.3±1.1
MCH pg	12.9-18.1	14.43±1.5	17.67±0.93	16.3±1.69	15.47±1.36	15.43±0.85
MCHC g/dl	21.9-33.5	31.1±0.26	31.2±0.1	30.87±0.25	29.6±0.43	30.5±0.2
RDW %	14.7-19.1	14.9±0.26	16.1±0.1	15.3±0.1	15.8±0.1	14.7±0.17
PLT* 10^3/µl	469-2364	295.67±13.32[a]	281.33±20.26[a]	310.13±24.58[a]	363.67±16.17[a]	283.67±14.5[a]
MPV fl	4.4-6.2	5.27±0.31	5.33±0.21	5.6±0.1	5.4±0.1	5.27±0.15
PDW %	-	14.97±0.55	15.67±0.38	14.93±0.35	15.9±0.2	15.03±0.21
PCT %	-	0.2±0.03	0.21±0.05	0.2±0.01	0.23±0.02	0.2±0.01

WBC, White Blood Cells; Lymph, Lymphocytes; Mon, Monocytes; Gran, Granulocytes; RBC, Red Blood Cells; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Blood Cells Distribution Width; PLT, Platelets; MPV, Mean Platelet Volume; PDW, Platelet Distribution Width; PCT, Plateletcrit.

[a] means < lower limit of reference range.

Table 5.2: Hematological parameters of mice treated with CuO-MSNs for 14 days (Acute toxicity)

Parameters	Range	Blank	Dose of CuO-MSNs (mg/kg/mice)			
		0	5	10	20	40
WBC * 10^3	4.58-16.21	5.47±1.41	5.9±0.44	5.7±0.53	5.4±0.26	6.17±0.12
Lymph* 10^3/µl	2.68-11.64	3.93±0.96	4.7±0.17	4.27±0.21	3.8±0.26	4.67±0.32
Mon* 10^3/µl	0-1.49	0.17±0.06	0.13±0.06	0.2±0.17	0.23±0.06	0.2±0.10
Gran* 10^3/µl	0.55-4.53	1.37±0.64	1.07±0.21	1.25±0.21	1.37±0.21	1.3±0.20
Lymph%	43.35-86.46	72.16±5.8	79.66±2.9	74.85±4.75	70.37±4.30	75.68±4.49
Mon%	0-13.44	3.35±1.72	2.26±0.78	3.51±1.88	4.36±1.32	3.26±1.67
Gran%	7.48-	24.5±6.51	17.98±2.1	22.17±2.19	25.28±3.31	21.09±3.30

	50.04		5			
RBC* 10^6	7.17-11.35	7.37±0.15	7.43±0.15	8.27±0.17	8.03±0.15	8.07±0.25
HGB g/dl	11.2-17.8	13.2±0.89	11.47±0.15	12.6±0.36	12.47±0.40	12.3±0.36
HCT%	38.2-64	42.77±2.95	47.2±0.56	41.5±0.72	43.03±1.40	39.83±0.32
MCV fl	47.5-66.7	48.03±0.67	50.1±0.69	53.8±0.72	51.6±0.53	49.6±0.84
MCH pg	12.9-18.1	14.43±1.50	15.37±1.23	13.43±1.17	15.47±0.73	15.23±0.83
MCHC g/dl	21.9-33.5	31.1±0.26	30.77±0.75	30±0.81	27.97±1.36	30.83±0.61
RDW %	14.7-19.1	14.9±0.26	12.5±0.38 [a]	14.4±0.26 [a]	15.2±0.26	16.47±0.38
PLT* 10^3/µl	469-2364	295.67±13.32 [a]	302±7.21 [a]	349.33±13.32 [a]	305.67±20.43 [a]	381.67±17.62 [a]
MPV fl	4.4-6.2	5.27±0.31	5.3±0.10	5.1±0.21	5.2±0.10	5.43±0.15

PDW %		14.97±0.55	14.95±0.38	14.45±0.35	14.8±0.35	15.23±0.20
PCT %		0.2±0.03	0.16±0.02	0.18±0.02	0.20±0.01	0.19±0.03

WBC, White Blood Cells; Lymph, Lymphocytes; Mon, Monocytes; Gran, Granulocytes; RBC, Red Blood Cells; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Blood Cells Distribution Width; PLT, Platelets; MPV, Mean Platelet Volume; PDW, Platelet Distribution Width; PCT, Plateletcrit.
[a] means < lower limit of reference range.

5.4.2.2 Sub-acute toxicity:

These studies provide information on organ toxicity and bioaccumuation potential of particles and designed to determine the no-observed-adverse-effect levels (NOAELs). The body weights of all mice were monitored daily till the end of treatment. Compared with the control mice, the body weights of all treated mice were similar, indicating a no significant toxicity even at the highest dose (1mg mouse^{-1} d^{-1}), as shown in Figure 5.4 (MSNs) and 5.5 (CuO-MSNs). No mice was found to show any unusual response, signs of infection or impaired mobility. The BCS of all mice were 3, confirming all mice were well conditioned according to the published scale.

Hematological parameters in the blood as described by the autoanalyzer are shown in Table-5.3 (MSNs) and Table 5.5 (CuO-MSNs). As seen in acute toxicity, here also all the mice, including control group, were found to exhibit low platelet count. Four mice, among which two mice treated with 5 mg/kg and 20 mg/kg of MSNs and two mice treated with 5 mg/kg and 40 mg/kg of CuO-MSNs showed little decrease in %RDW (12.3, 13.8 and 12.6, 13.7 respectively; Normal range: 14.7-19.1%). Low % RDW, just indicates a uniformity in the size of red blood cells. One mice treated with 20 mg/kg of CuO-MSNs showed very little decrease in mean corpuscular volume (MCV). This might be due to poor iron intake or menstrual bleeding.

Biochemical parameters of the blood serum are represented in Table-5.4 (MSNs) and Table 5.6 (CuO-MSNs). One mice treated with 20 mg/kg of MSNs also showed little decrease in total bilirubin (0.07 mg/dl; Normal range: 0.1-0.4 mg/dl) which is usually not a concern regarding health. One mice treated with 5 mg/kg of CuO-MSNs showed little decrease in cholesterol level (82 mg/dl; Normal range: 85-244 mg/dl). Low cholesterol level is usually considered better.

Representative histopathological images of major organs are showed in Figure 5.6. One of the control group mouse showed mild hypertrophy of hepatocyes. Though it was observed in control group mouse, it has no correlation with administration of MSNs. One of the control group mouse and one mouse treated with 10 $mg.kg^{-1}$ of MSNs showed minimal multifocal lymphocytic infilteration. This mild change may result due to stress also. Mild congestion in lung of one of the mouse treated with 5 $mg.\ kg^{-1}$ of CuO-MSNs was observed but congestion in lung is not a pathological.

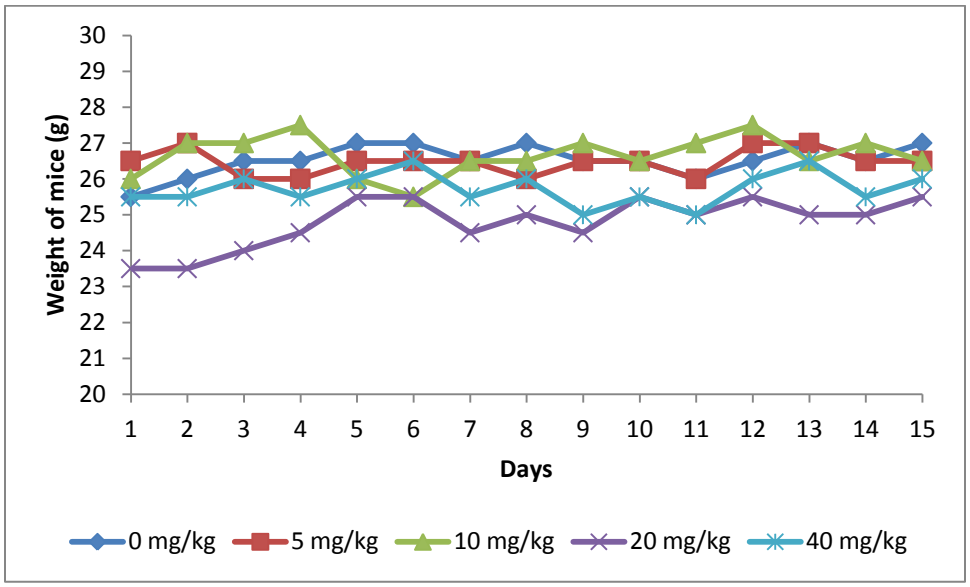


Figure 5.4: Average body weight of mice treated with MSNs for 14 days (Sub-acute toxicity).

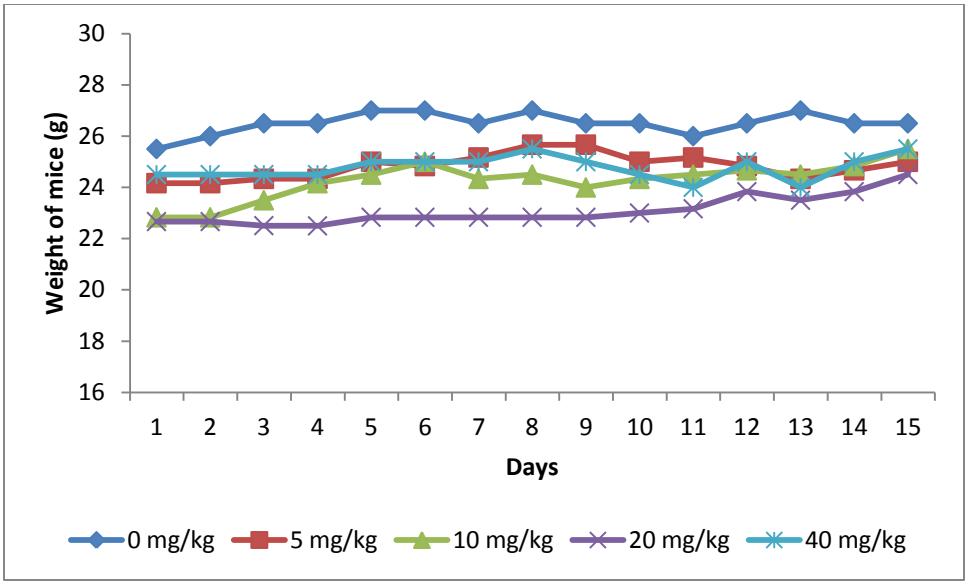


Figure 5.5: Average body weight of mice treated with CuO-MSNs for 14 days (Sub-acute toxicity).

Table 5.3: Hematological parameters of mice treated with MSNs for 14 days (Sub-acute toxicity).

Parameters	Reference Range	Control	Dose of MSNs (mg/kg/mice)			
		0	5	10	20	40
WBC * 10^3	4.58-16.21	8.97±0.71	8.17±0.64	10.67±0.71	7.33±0.81	11.33±0.31
Lymph* 10^3/µl	2.68-11.64	6.8±0.74	5.67±0.36	7.67±0.45	6.07±0.38	6.67±0.83
Mon* 10^3/µl	0-1.49	0.3±0.1	0.27±0.06	0.47±0.06	0.07±0.06	0.8±0.1

Gran* 10^3/µl	0.55-4.53	1.87±0.32	2.13±0.21	2.53±0.29	1.2±0.4	3.87±0.7
Lymph%	43.35-86.46	75.84±2.05	69.39±3.48	71.88±0.71	82.73±3.41	58.82±6.67
Mon%	0-13.44	3.35±1.02	3.30±0.92	4.38±0.67	1.91±0.84	7.06±0.64
Gran%	7.48-50.04	20.82±2.1	26.12±3.18	23.75±1.4	15.36±2.1	34.12±3.3
RBC* 10^6	7.17-11.35	8.8±0.36	7.83±0.55	8.1±0.44	7.27±0.31	8.4±0.53
HGB g/dl	11.2-17.8	13±0.79	11.6±0.56	11.7±0.7	11.2±0.87	12.8±0.7
HCT%	38.2-64	40.8±1.28	43.13±1.68	39.4±0.75	41.1±0.87	40.2±0.98
MCV fl	47.5-66.7	49±1.54	50.1±0.53	55.1±1.82	49.5±1.84	50.47±1.01
MCH pg	12.9-18.1	15.73±0.71	16.1±1.25	17.57±0.85	16.27±0.85	16.37±0.55
MCHC g/dl	21.9-33.5	31.93±0.15	32.2±0.1	31.87±0.06	32.6±0.06	32.47±0.1
RDW %	14.7-19.1	15.27±0.31	12.3±0.7[a]	18.7±0.26	13.8±0.56[a]	17.57±0.35
PLT* 10^3/µl	469-2364	398±31.1	386.67±16.5	406±27.0	363.67±16.2	420.5±20.5

		9[a]	6[a]	7[a]	6[a]	3[a]
MPV fl	4.4-6.2	5.43±0.25	6.33±0.21	5.1±0.2	5.1±0.46	5.07±0.21
PDW %	-	15.23±0.64	15.93±0.45	14.65±0.6	14.9±0.36	14.5±0.3
PCT %	-	0.24±0.05	0.31±0.01	0.21±0.04	0.26±0.01	0.26±0.01

WBC, White Blood Cells; Lymph, Lymphocytes; Mon, Monocytes; Gran, Granulocytes; RBC, Red Blood Cells; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Blood Cells Distribution Width; PLT, Platelets; MPV, Mean Platelet Volume; PDW, Platelet Distribution Width; PCT, Plateletcrit.
[a] means < lower limit of reference range.

Table 5.4: Biochemical parameters of mice treated with MSNs for 14 days (Sub-acute toxicity).

Biochemical Parameters						
Parameters	**Range**	**Blank**	**Dose of MSNs (mg/kg/mice)**			
		0	**5**	**10**	**20**	**40**
Creatinine (mg/dl)	0.0-0.4	0.4±0.01	0.3±0.01	0.33±0.06	0.3±0.01	0.37±0.03
BUN (mg/dl)	9-24	17.5±0.23	21.67±0.36	20±0.24	20.67±0.26	18±0.31
T. Protein (g/dl)	4.7-6.6	5.77±0.06	5.97±0.04	5.9±0.08	6.03±0.04	6.1±0.06

T. Bilirubin (mg/dl)	0.1-0.4	0.05±0.01[a]	0.13±0.02	0.13±0.01	0.07±0.01[a]	0.17±0.02
Glucose (mg/dl)	137-319	132±11.53[a]	134±6.08[a]	124±4.36[a]	122.33±4.73[a]	137±5.57
Albumin (g/dl)	2.6-4.1	3.33±0.03	3.1±0.06	3.67±0.03	3.17±0.04	3.67±0.03
Cholesterol (mg/dl)	85-244	88.67±1.28	86.33±1.26	88±1.81	98.33±1.62	98.67±1.34
AST (U/l)	45-182	112.6±11.34	82.56±5.59	87.30±9.87	96.62±13.2	73.33±9.6
ALT (U/l)	18-71	35.45±2.32	27.40±1.81	28.70±2.16	24.76±1.21	20.26±1.67
GGT (U/l)	0-19	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00

BUN, Blood urea nitrogen; T.Protein, Total Protein; T.Bilirubin, Total Bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase, GGT, gamma-glutamyl transferase.
[a] means < lower limit of reference range.

Table 5.5: Hematological parameters of mice treated with CuO-MSNs for 14 days (Sub-acute toxicity).

Parameters	Range	Blank	Dose of CuO-MSNs (mg/kg/mice)			
		0	5	10	20	40
WBC * 10^3	4.58-16.21	8.97±0.71	8.33±0.55	8.13±0.57	9.07±1.19	8.8±0.56
Lymph* 10^3/µl	2.68-11.64	6.8±0.74	6.53±0.71	5.37±0.70	6.6±0.45	7.03±0.81
Mon* 10^3/µl	0-1.49	0.3±0.1	0.23±0.11	0.17±0.14	0.37±0.22	0.33±0.28
Gran* 10^3/µl	0.55-4.53	1.87±0.32	1.57±0.37	2.59±0.46	2.1±0.25	1.44±0.34
Lymph%	43.35-86.46	75.84±2.05	78.39±1.89	66.05±2.23	72.77±3.12	79.89±1.86
Mon%	0-13.44	3.35±1.02	2.76±0.57	2.09±0.75	4.08±1.14	3.75±1.36
Gran%	7.48-50.04	20.82±2.1	18.85±1.82	31.86±2.36	23.15±1.68	16.36±1.31
RBC* 10^6	7.17-11.35	8.8±0.36	8.00±0.34	7.94±0.26	7.43±0.22	7.87±0.31

HGB g/dl	11.2-17.8	13±0.79	13±0.89	12.57±0.72	11.33±1.12	11.53±0.46
HCT%	38.2-64	40.8±1.28	40.2±1.12	38.8±1.36	39.17±0.96	38.67±0.84
MCV fl	47.5-66.7	49±1.54	50.27±2.14	48.9±1.62	47.33±1.34 [a]	49.13±1.58
MCH pg	12.9-18.1	15.73±0.71	16.2±0.51	15.77±0.56	15.2±0.36	14.57±0.84
MCHC g/dl	21.9-33.5	31.93±0.15	32.3±0.17	32.3±0.23	32.17±0.23	32.3±0.17
RDW %	14.7-19.1	15.27±0.31	12.6±0.33 [a]	14.07±0.22	14.7±0.46	13.7±0.21 [a]
PLT* 10^3/µl	469-2364	398±31.19 [a]	462.33±17.43 [a]	360.67±23.36 [a]	466±34.87 [a]	487.67±28.64 [a]
MPV fl	4.4-6.2	5.43±0.25	5.63±0.23	5.73±0.51	5.63±0.42	5.57±0.31
PDW %		15.23±0.64	15.37±0.53	15.33±0.56	15.13±0.47	15.17±0.61
PCT %		0.24±0.05	0.26±0.03	0.21±0.02	0.26±0.04	0.27±0.03

WBC, White Blood Cells; Lymph, Lymphocytes; Mon, Monocytes; Gran, Granulocytes; RBC, Red Blood Cells; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume;

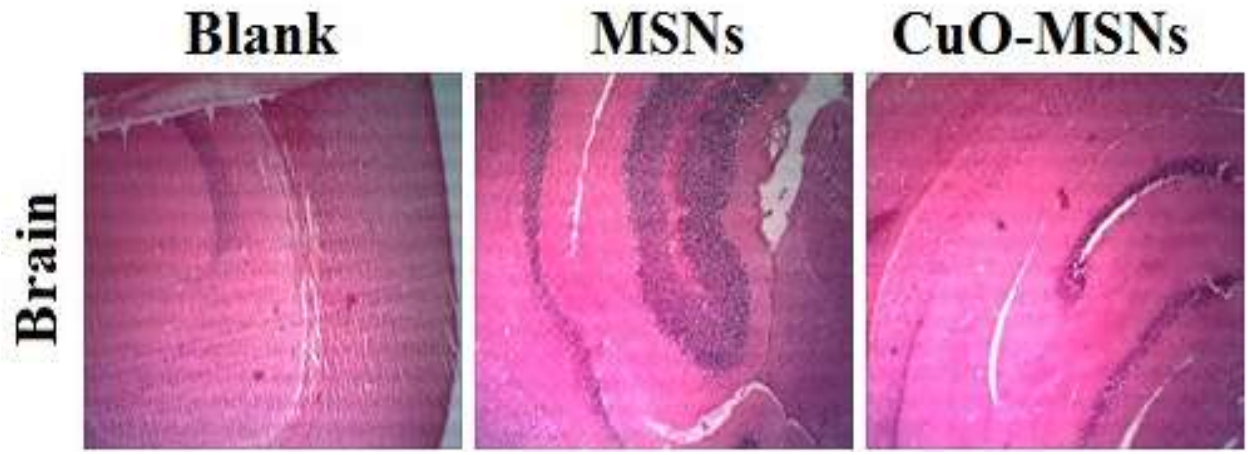
MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Blood Cells Distribution Width; PLT, Platelets; MPV, Mean Platelet Volume; PDW, Platelet Distribution Width; PCT, Plateletcrit.
[a] means < lower limit of reference range.

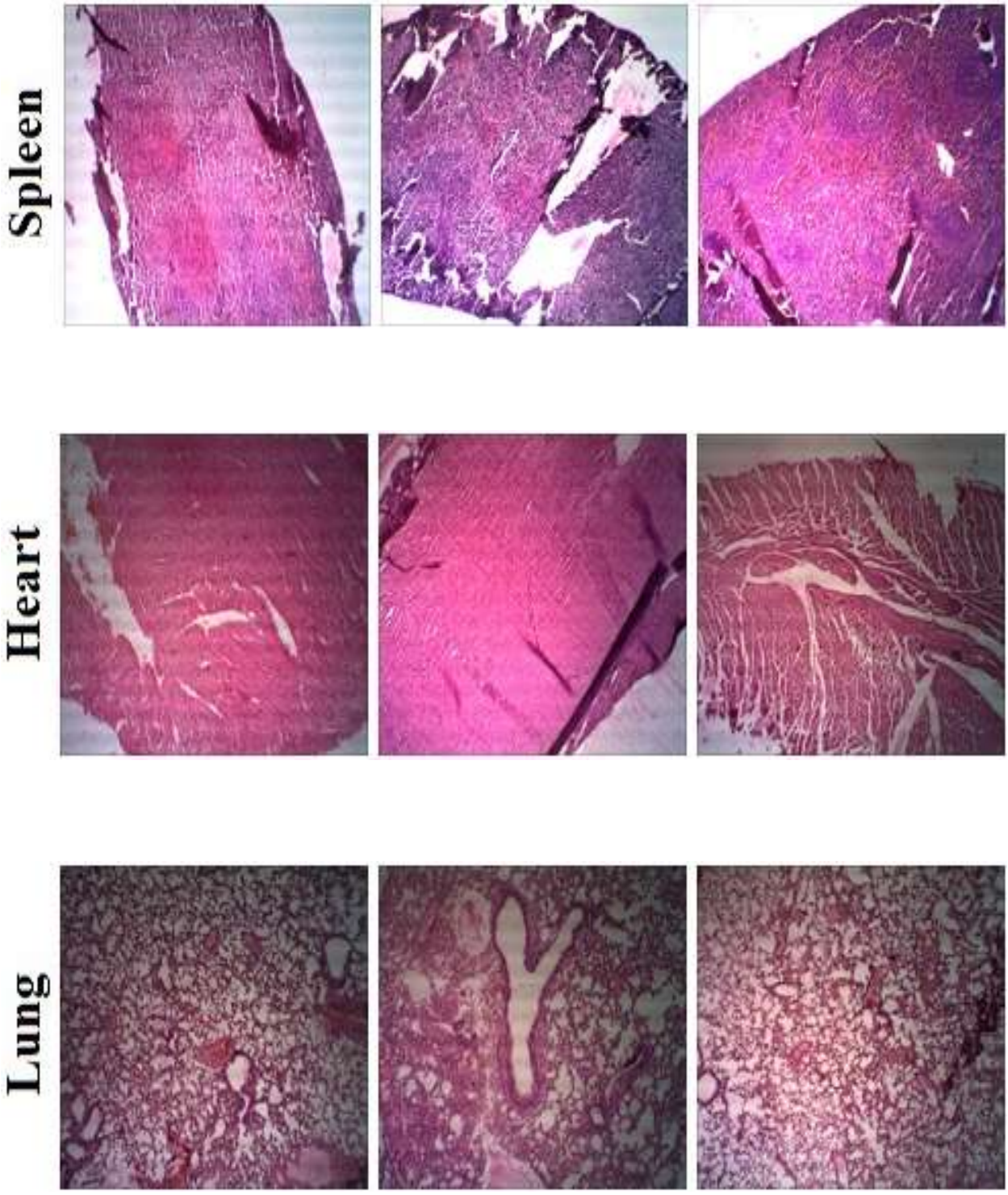
Table 5.6: Biochemical parameters of mice treated with CuO-MSNs for 14 days (Sub-acute toxicity).

Biochemical Parameters						
Parameters	**Range**	**Blank**	**Dose of CuO-MSNs (mg/kg/mice)**			
		0	**5**	**10**	**20**	**40**
Creatinine (mg/dl)	0.0-0.4	0.4±0.01	0.37±0.03	0.33±0.03	0.43±0.01	0.4±0.01
BUN (mg/dl)	9-24	17.5±0.23	22±0.12	21.33±0.21	25.67±0.36	32±0.24
T. Protein (g/dl)	4.7-6.6	5.77±0.06	5.63±0.07	5.33±0.06	5.9±0.04	5.47±0.04
T. Bilirubin (mg/dl)	0.1-0.4	0.05±0.01[a]	0.17±0.04	0.2±0.06	0.13±0.02	0.03±0.01[a]
Glucose (mg/dl)	137-319	132±11.53[a]	144.67±10.89	139±7.64	152.67±9.31	137.67±6.13
Albumin (g/dl)	2.6-	3.33±0.03	3.33±0.05	3.27±0.03	3.27±0.02	3.07±0.02

	4.1					
Cholesterol (mg/dl)	85-244	88.67±1.28	82±1.02 [a]	89±1.08	85±0.98	88±0.86
AST (U/l)	45-182	112.6±11.34	94.85±8.63	121.06±9.41	79.76±10.68	138.17±12.23
ALT (U/l)	18-71	35.45±2.32	24.76±2.14	30.38±2.36	31.51±1.89	37.99±2.64
GGT (U/l)	0-19	0±0.00	0±00	0±00	0±00	0±00

BUN, Blood urea nitrogen; T.Protein, Total Protein; T.Bilirubin, Total Bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase, GGT, gamma-glutamyl transferase.
[a] means < lower limit of reference range.

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Heart
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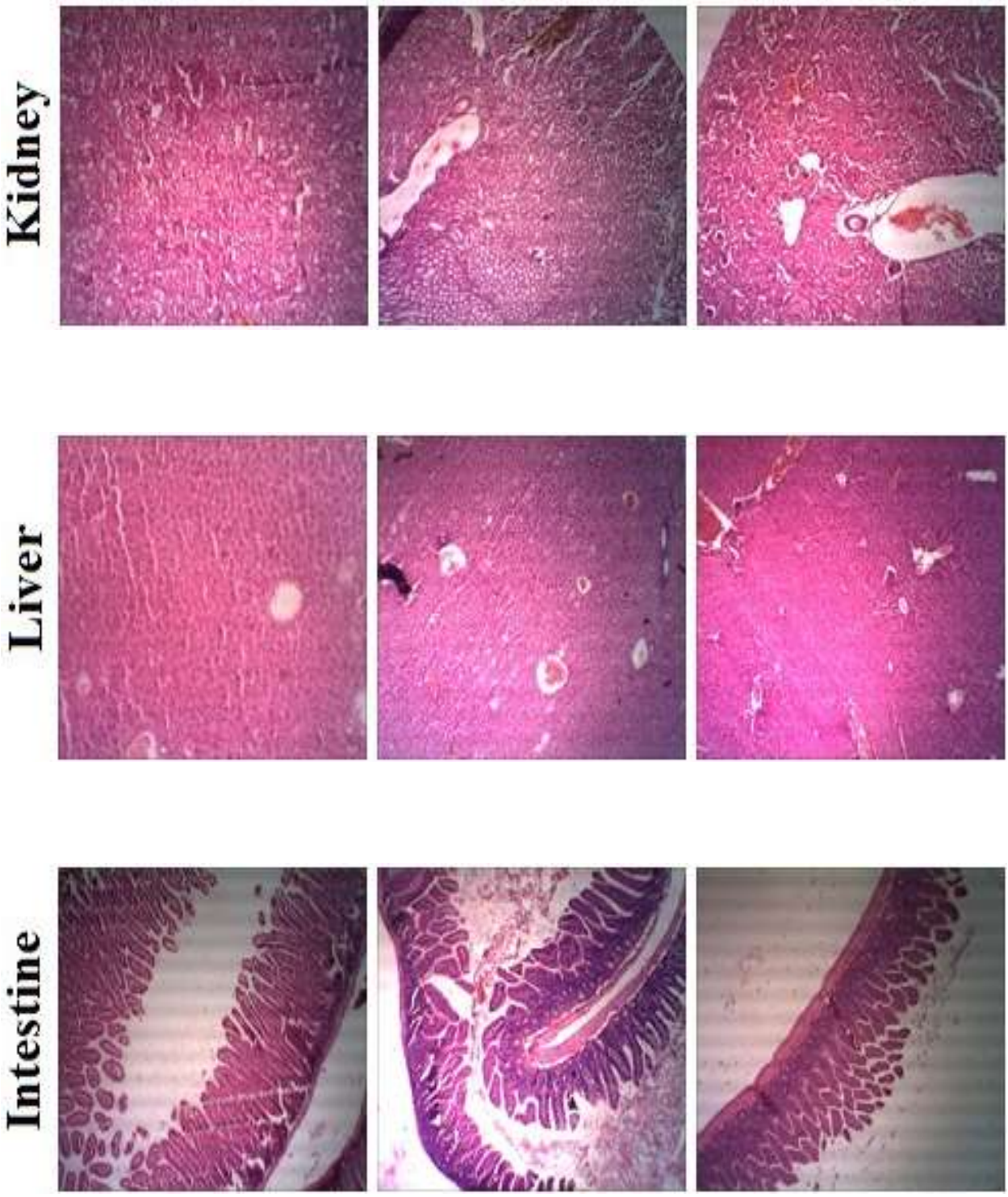


Figure 5.6: Histopathological examination representative of H&E staining of major organs after treatment with MSNs and CuO-MSNs for 14 days (Sub-acute toxicity).

5.4.2.3 Chronic toxicity:

The body weights of all mice were monitored daily up to 60 days. Compared with the control mice, the body weights of all treated mice were similar, indicating a no significant toxicity (Figure 5.7). No mice was found to show any unusual response, signs of infection or impaired mobility. The BCS of all mice were 3, confirming all mice were well conditioned according to the published scale. The results for the hematological parameters after treatment with MSNs and CuO-MSNs for 60 days are shown in Table 5.7. None of the hematological parameter was found to be out of the range in treatment group, signifying that neither MSNs nor CuO-MSNs induced any hematological toxicity at administered dose. Table 5.8 represents biochemical parameters of mice blood after treatment with MSNs and CuO-MSNs for 60 days. Various biochemical parameters in serum were also found within the range for all mice which confirmed that at administered dosage MSNs did not cause any organ specific toxicity. Figure 5.8 represents the histopathology of major organs of mice. At the end of the treatment, no noticeable histopathological abnormalities or lesions with any of the tissues, neither gross nor pathological abnormality related to treatment were observed in major organs, such as the liver, spleen, kidney, heart, intestine, lungs and brain.

The observed results suggests that the synthesized MSNs and CuO-MSNs are safe for pharmacological application up to 40 $mg.kg^{-1}$ dose.

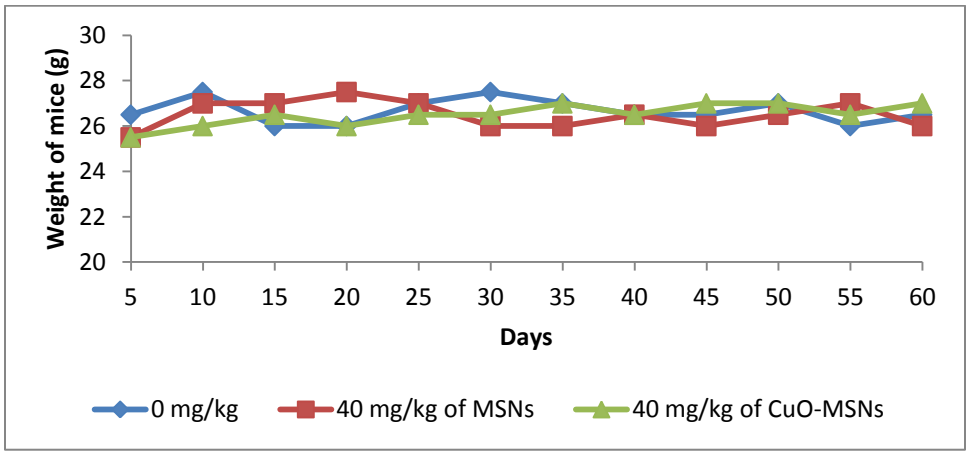


Figure 5.7: Average body weight of mice treated with MSNs and CuO-MSNs for 60 days (Chronic toxicity).

Table 5.7: Hematological parameters of mice treated with MSNs and CuO-MSNs for 60 days (Chronic toxicity).

Parameters	Range	Blank	MSNs	CuO-MSNs
WBC * 10^3	4.58-16.21	7.4±0.83	8.3±0.17	5.8±0.67
Lymph* 10^3/µl	2.68-11.64	5.13±0.95	6.63±0.51	4±0.42
Mon* 10^3/µl	0-1.49	0.2±0.1	0.23±0.06	0.17±0.03
Gran* 10^3/µl	0.55-4.53	2.07±0.78	1.43±0.35	1.63±0.14
Lymph%	43.35-86.46	67.77±4.34	79.73±4.5	68.6±5.60
Mon%	0-13.44	3.3±0.8	2.7±0.64	3.27±0.74
Gran%	7.48-50.04	28.9±3.50	17.6±4.40	28.13±2.81
RBC* 10^6	7.17-11.35	8.67±0.96	8.32±0.19	8.57±0.84
HGB g/dl	11.2-17.8	13.1±0.53	12.33±0.48	13.63±0.56
HCT%	38.2-64	41.33±1.18	39.17±1.53	40.8±1.23
MCV fl	47.5-66.7	48.03±3.92	47.1±0.89	47.63±2.36
MCH pg	12.9-18.1	15.13±1.15	14.77±0.25	15.87±0.83

MCHC g/dl	21.9-33.5	31.67±1.00	31.43±0.32	33.43±0.07
RDW %	14.7-19.1	13.4±1.11[a]	15.13±1.03	12.4±1.16 [a]
PLT* 10^3/µl	469-2364	391±52.2[a]	360.33±43[a]	409.67±46.72 [a]
MPV fl	4.4-6.2	5.27±0.06	5.23±0.15	5.03±0.26
PDW %		15±0.26	14.77±0.15	14.77±0.23
PCT %		0.21±0.03	0.19±0.02	0.23±0.02

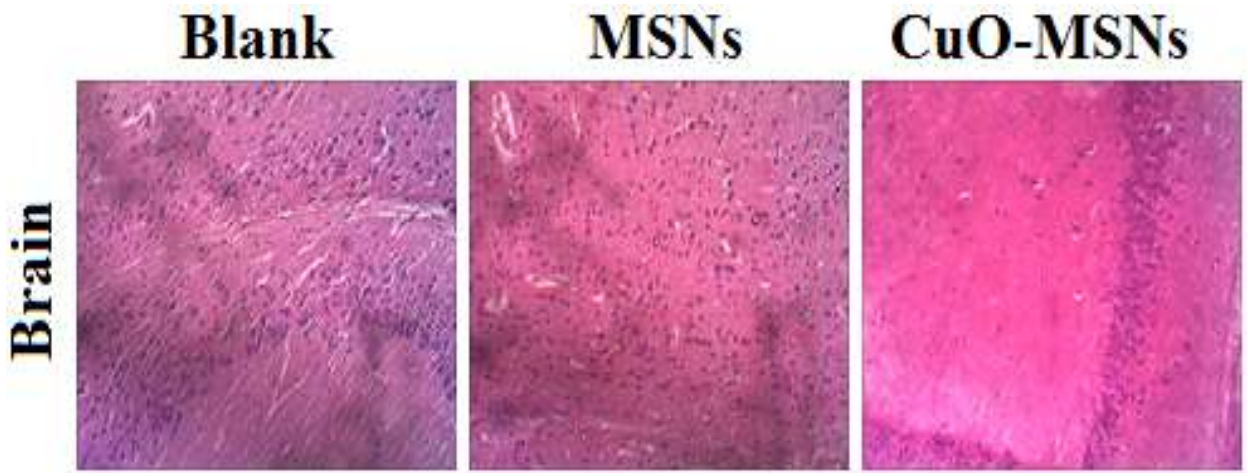
WBC, White Blood Cells; Lymph, Lymphocytes; Mon, Monocytes; Gran, Granulocytes; RBC, Red Blood Cells; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Blood Cells Distribution Width; PLT, Platelets; MPV, Mean Platelet Volume; PDW, Platelet Distribution Width; PCT, Plateletcrit.
[a] means < lower limit of reference range.

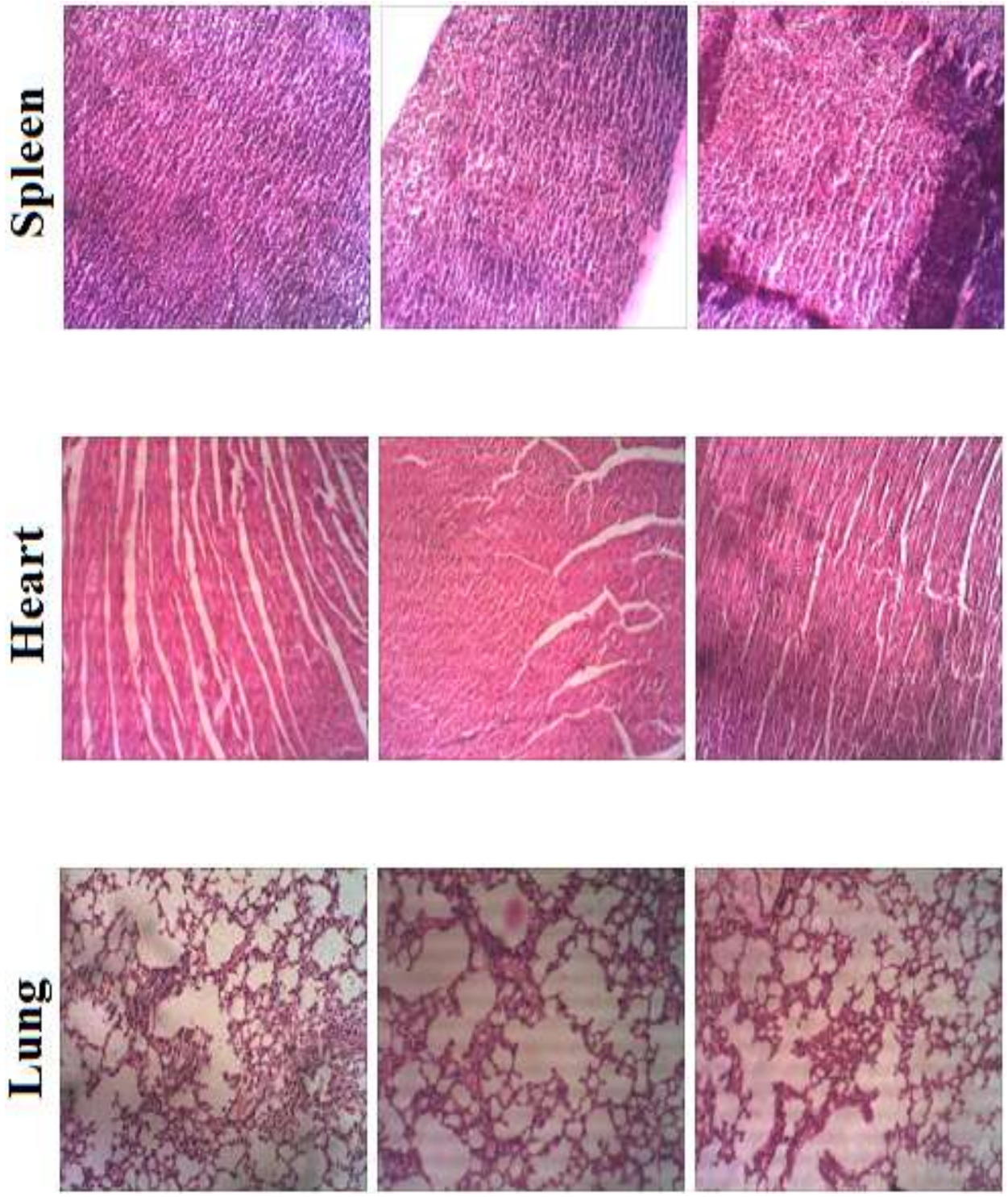
Table 5.8: Biochemical parameters of mice treated with MSNs and CuO-MSNs for 60 days (Chronic toxicity).

Biochemical parameters				
Parameters	**Range**	**Blank**	**MSNs**	**CuO-MSNs**
Creatinine (mg/dl)	0.0-0.4	0.3±0.01	0.3±0.02	0.2±0.01
BUN (mg/dl)	9-24	18.5±0.31	18±0.23	20.2±0.36
T. Protein (g/dl)	4.7-6.6	5.6±0.09	6.1±0.07	5.7±0.1

T. Bilirubin (mg/dl)	0.1-0.4	0.1±0.01	0.1±0.01	0.1±0.01
Glucose (mg/dl)	137-319	151±14.56	184±15.68	176±15.42
Albumin (g/dl)	2.6-4.1	3.2±0.02	3.4±0.02	3.3±0.03
Cholesterol (mg/dl)	85-244	118±9.86	114±8.61	131±10.27
AST (U/l)	45-182	103.89±8.14	98.25±6.42	141.69±7.68
ALT (U/l)	18-71	33.17±2.23	29.33±1.67	27.93±1.34
GGT (U/l)	0-19	0±0.00	0±0.00	0±0.00

BUN, Blood urea nitrogen; T.Protein, Total Protein; T.Bilirubin, Total Bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase, GGT, gamma-glutamyl transferase.
[a] means < lower limit of reference range.

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Heart
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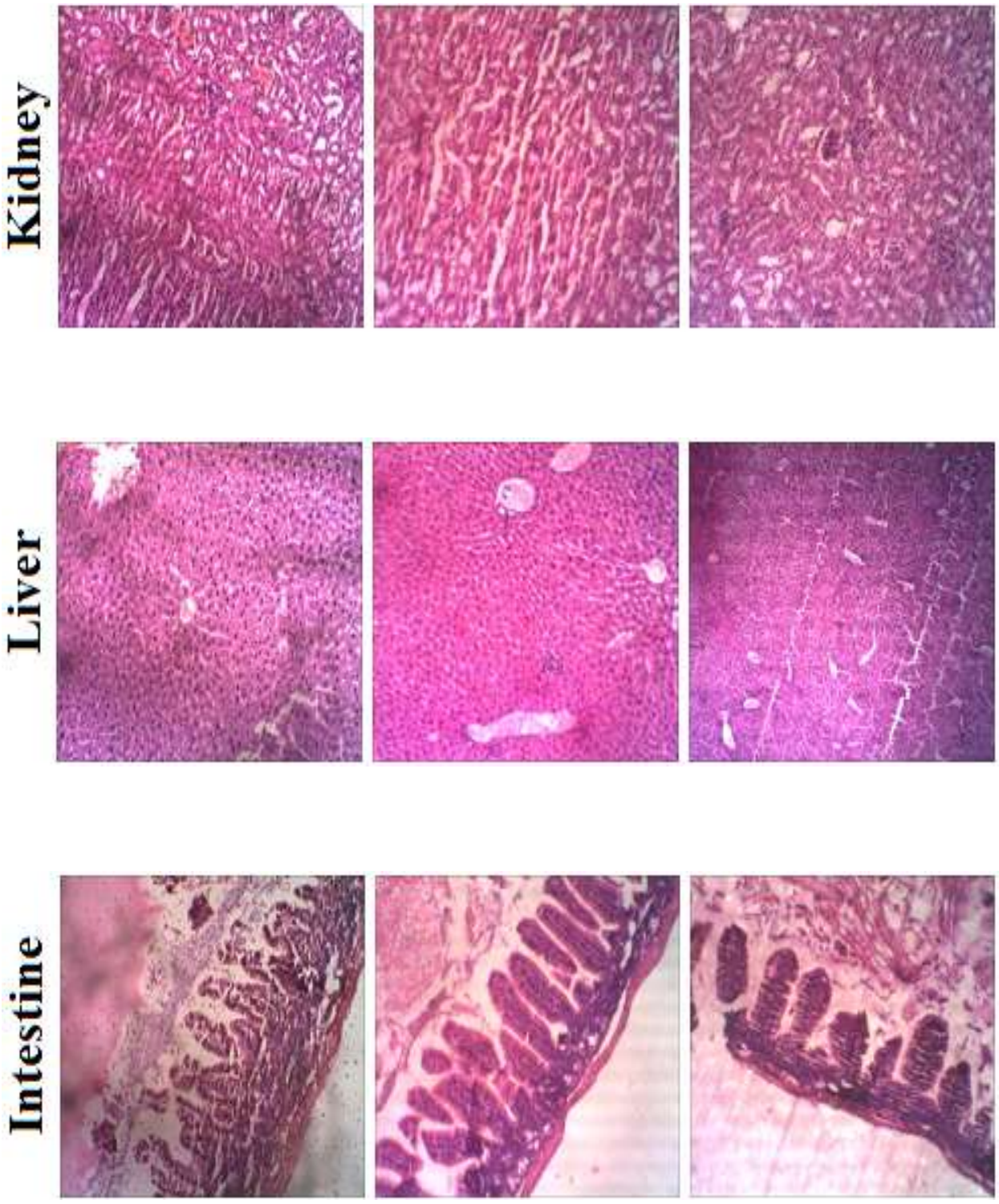

Figure 5.8: Histopathological examination representative of H&E staining of major organs after treatment with MSNs and CuO-MSNs for 60 days (Chronic toxicity).

5.4.3 Degradation of MSNs:

5.4.3.1 In vitro degradation:

The in vitro degradation behavior of both MSNs and CuO-MSNs, synthesized using commercial sodium silicate, was measured in PBS (pH 7.4). As shown in Figure 5.9 and 5.10, respectively. The degradation rate of MSNs as well as CuO-MSNs was faster initially which decreases gradually. This might be due to increase in the silicic acid concentration in surrounding medium. Both nanoparticles were found to be dissolved completely in the PBS 7.4 within 6 days.

5.4.3.2 In vivo degradation:

MSNs are known to excreted out of body as soluble silicic acid by urine and feces. Hence, the in vivo degradation of MSNs and CuO-MSNs, synthesized using commercial sodium silicate, was calculated by measuring the amount of silicic acid in the urine and feces of wistar rats injected with nanoparticles. As shown in Figure 5.9, the MSNs were found to be completely excreted out of the body within 3-4 days. Similarly, CuO-MSNs were also completely excreted out of the body within 3-4 days (Figure 5.10). Rate of excretion of silica was almost constant and did not decrease with the time. This might be due to continuous elimination of soluble silicic acid from the body.

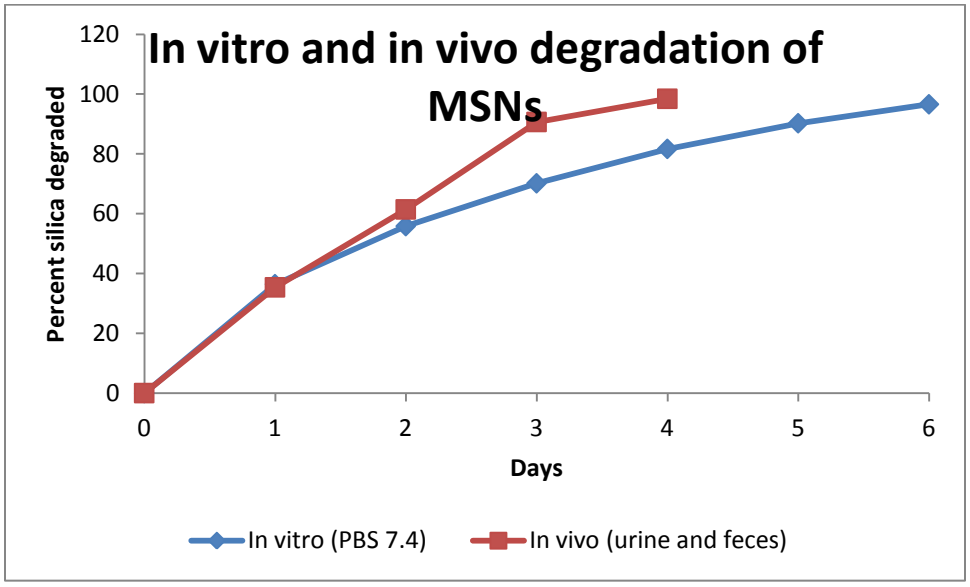

Figure 5.9: Degradation of MSNs in vitro (PBS 7.4) and in vivo (urine and feces).

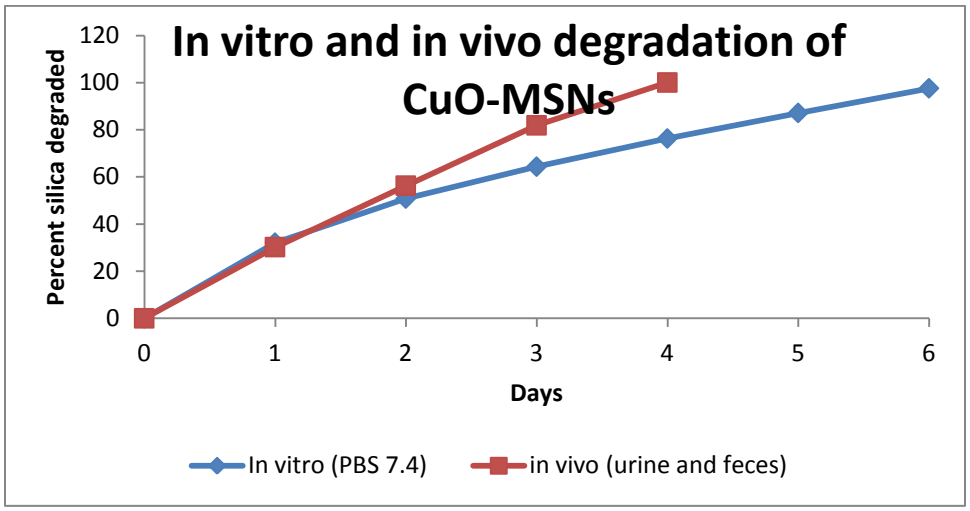

Figure 5.10: Degradation of CuO-MSNs in vitro (PBS 7.4) and in vivo (urine and feces).

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