8. IN VIVO ANTICANCER STUDIES

Table 8.1: List of Chemicals.

Sr. No.	Chemicals	Source and Place
1	Doxorubicin Hydrochloride	Gift sample from Sun Pharmaceuticals, India
2	Dulbecco's Modified Eagle Medium (DMEM)	Himedia, India
3	Fetal Bovine Serum (FBS)	Himedia, India
4	Trypsin-EDTA solution	Himedia, India
5	Antibiotic / Antimycotic solution (Penicillin G, Streptomycin and Amphotericin B)	Himedia, India
6	Trypan blue dye	Himedia, India
7	Isopropyl alcohol	Himedia, India
8	Sodium chloride injection IP (0.9% w/w)	Claris-Otsuka Pvt. Ltd., India
9	Dipotassium ethylenediaminetetraacetic acid (K_2 EDTA)	Fisher Scientific, India
10	Diethyl ether	Rankem, India
11	Reagent kits for various hepatic parameters	Span Diagnostics Pvt. Ltd., India

12	Reagent kits for various renal parameters	Span Diagnostics Pvt. Ltd., India
13	Reagent kits for cardiac marker CK-MB	Coral Clinical Systems, India

Table 8.2: List of Instruments.

Sr. no.	**Equipment**	**Company Name and Place**
1	Digital weighing machine AX 120	Shimadzu, Japan
2	Bath Sonicator	Fast clean ultra-cleaner, India
3	Incubator	JGUAN quality system, India
4	Laminar Air Flow	Weiber, India
5	Inverted Microscope	Nikon, USA
6	Centrifuge	Remi, India
7	Auto Hematology Analyzer BC-2800V	Mindray, China
8	UV-Vis spectrophotometer 1800	Shimadzu, Japan
9	Labomed Vision 2000 binocular Microscope	Labomed, USA

8.1 Introduction:

Though the in vitro cell line screening techniques provide faster results in a cost effective manner, only cytotoxic compounds are screened by these methods. Apart from cytotoxicity, the pharmacokinetics and toxicity evaluations are also important with a view to measure complete effectiveness of a compound. Cell line studies are inadequate to estimate the off-target effects which may contribute to the potency or toxicity of the novel formulation.[1] Hence, the drug candidate/formulation progresses to in vivo animal testing after completion of the initial in vitro cell line studies. Animal studies are critical for understanding the fundamental processes that support in vivo tumor development as tumor cells grown in vitro are not necessarily analogous to those that develop in a human subject.[2]

Among various models of cancer research, mouse cancer models are well known and commonly used as models for cancer research.[3] The mouse has been the traditional animal model for basic and preclinical studies of cancer because it has number of advantages such as:

- The similarity of human and mouse genomes;
- The low cost of housing and maintenance;
- The short gestation period and rapid reproduction rate; and
- The rapid growth rate of implanted tumors.[2]

Variety of preclinical murine models of cancer have been developed (eg. xenografts, genetically engineered, and syngeneic mice) to study the development and progression of cancer as well as to increase the understanding of the etiology and dissemination of cancer in order to overcome barriers to early detection and resistance to standard chemotherapy.[4] Syngeneic tumor models, also known as allograft mouse tumor models, are tumor models whose genetic background is similar, if not identical, to the host animal.[5] A mouse tumor growing in mice of the strain in which the tumor originated, offer several advantages such as:

- Relatively low cost and high reproducibility.
- Grow in immuno-competent hosts.
- Wide variety of tumor types.

- Generally non-immunogenic.
- Long history of use and strong baseline of drug response data.
- Hosts are readily available.
- Studies are easily conducted with statistically meaningful numbers of mice per group.

Mouse or rat (murine) cancer cell line or tissues are transplanted in animals of same species due to which lack of transplant rejection by the immune system of host is observed. This permits researchers to monitor the tissues changes, such as growth or shrinkage, metastasis, and survival rate. Therapeutic interventions can be performed and the results are assessed to understand the treatment potentials.[6]

For the purpose of in vivo modeling, experimental tumors are of great importance and Ehrlich ascites carcinoma (EAC) is one of the most frequently used among them. It was discovered as a spontaneous breast cancer in a female mouse and then Ehrlich and Apolant used it as an experimental tumor by transplanting tumor tissues subcutaneously from mouse to mouse. EAC is an undifferentiated carcinoma, originally hyperdiploid with high transplantable capability, no-regression, rapid proliferation, shorter life span and 100% malignancy.[7] For these reasons, in the present study, EAC was chosen as a rapidly growing experimental tumor model.

8.2 Cell line:

EAC cell line obtained from the National Centre for Cell Sciences (NCCS, Pune, India) was used for induction of tumor in vivo. It was grown in DMEM supplemented with L-glutamine (2 mM), 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (100 µg/ml streptomycin, 100 units/ml penicillin and 50 µg/ml amphotericin). The culture was maintained at 37 ºC under a humidified atmosphere with 5% CO_2. The culture medium was changed at regular time intervals.

8.3 Ethical Statement:

Total 24 female Balb C mice obtained from Sun Pharmaceutical Advanced Research Centre, India, were used for the study. All experiments have been conducted under the regulations and after the approval of the study protocol by Institutional Animal Ethics Committee

(IAEC). Animal care and handling throughout the experimental procedure were performed in accordance to the Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA) guidelines.

8.4 Animals:

Female mice were selected for the study as EAC cells are reported to show greater initial growth and total cell count in female than male mice.[8] Two to three weeks old female Balb C mice weighing 20-25 g were housed 8-10 per cage, and allowed to become acclimatized to laboratory conditions for two weeks before the experiment. The animals were kept in a conditioned atmosphere at 25 °C and fed with standard laboratory food and water.

8.5 Experimental design and tumor cell transplantation:

The mice were randomly allocated into eight groups: Normal control, model control, standard control, and test group I-V (n=3 animals for each group). The details of groups is shown in Table 8.3.

Table 8.3: Animal groups for in vivo anticancer study.

Groups		Administered samples
I	Normal Control	0.9% saline (No Tumor)
II	Model Control	0.9% saline (Tumor induced)
III	Standard control	Drug solution (2.5 mg/kg)
IV	Test group I	DOX-MSN (equivalent to 2.5 mg/kg of drug)
V	Test Group II	DOX-MSN-SS-CH-FA (equivalent to 2.5 mg/kg of drug)

VI	Test Group III	CuO-MSNs (2.5 mg/kg)
VII	Test Group IV	DOX-CuO-MSNs (equivalent to 2.5 mg/kg of drug)
VIII	Test Group V	DOX-CuO-MSN-SS-CH-FA (equivalent to 2.5 mg/kg of drug)

All the groups, except normal control, were injected with EAC cells (0.2ml of $1*10^6$ cells/mouse) sub-cutaneously in the abdomen region to cause mammary tumor. After 2 weeks of tumor inoculation, the animals were given intravenous dose (equivalent to 2mg/kg of DOX) of specified formulations as per their group, twice weekly up to 30 days. The parameters checked included, change in body weight, tumor size, hematological parameters, biochemical parameters to measure liver toxicity, renal toxicity and cardiac toxicity. Histopathological analysis of different organs such as mammary gland, liver, kidneys, lungs and heart was performed.

8.6 Measurement of tumor growth and Evaluation of toxicity:

8.6.1 Body weight change:

The day on which the animals were inoculated with the EAC cells was noted as day 0. Body weight of all the groups of experimental mice was recorded daily from the beginning of the experiment.

8.6.2 Tumor volume measurement:

Antitumor activity of different formulations was evaluated by measuring tumor growth inhibition. The tumor size was measured from the 10^{th} day of tumor inoculation and the measurement was carried out every 5^{th} day up to 30 days. The tumor volume was determined by the formula:

$$\text{Tumor volume} = L.W^2/2 \qquad (8.1)$$

Where, L is the length and W is the width of tumor mass.

8.6.3 Increase in the life span (ILS):

The survival of tumor-bearing mice was observed until the tumor volume reached 50 % above the ethical limit (2000 mm^3) or death occurred after initiation of the treatments; whichever event was first observed was counted as death. From the survival data, ILS was calculated according to the following formula:

$$\% \text{ ILS} = \left(\frac{T}{C} - 1\right) * 100 \qquad (8.2)$$

where T and C are the mean survival time of treated mice and control mice.

8.6.4 Toxicity profiling:

At the end of the experiment, the animals were anesthetized using diethyl ether, blood samples were collected from the retro-orbital plexus using heparinized syringes, transferred to eppendorf tubes containing K_2-EDTA solution and used for the estimation of complete blood count.

The blood was collected in separate eppendorf tubes and plasma was separated as supernatant by centrifugation at 3000 rpm for 10 min. The concentration of hepatic markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) were measured in plasma colorimetrically by using commercially available reagent kits purchased from Span Diagnostic Pvt. Ltd, India. Urea and creatinine were also determined in plasma colorimetrically using reagent kits purchased from Span Diagnostic Pvt. Ltd, India, in order to determine the proper functioning of kidneys. Creatine kinase-MB (CK-MB), a cardiac marker, level was assayed using the commercially available diagnostic kit purchased from Coral Clinical Synstems, India.

8.6.5 Histopathological studies:

After 30 days of the study, the animals were sacrificed by overdose of thiopentone sodium and tissue portions from the mammary gland, liver, kidneys, lungs and heart were collected from all the experimental groups, fixed 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.

8.7 Statistical analysis:

All the results are expressed as mean ± standard deviation. Statistical analysis. The data were analysed using GraphPad Prism 7 software. Multi-group comparisons of the means were carried out by one way analysis of variance (ANOVA) test. $P<0.05$ were considered statistically significant. Kaplan-Meier survival curve was also prepared to compare the survival of animals

8.8 Results and Discussions:

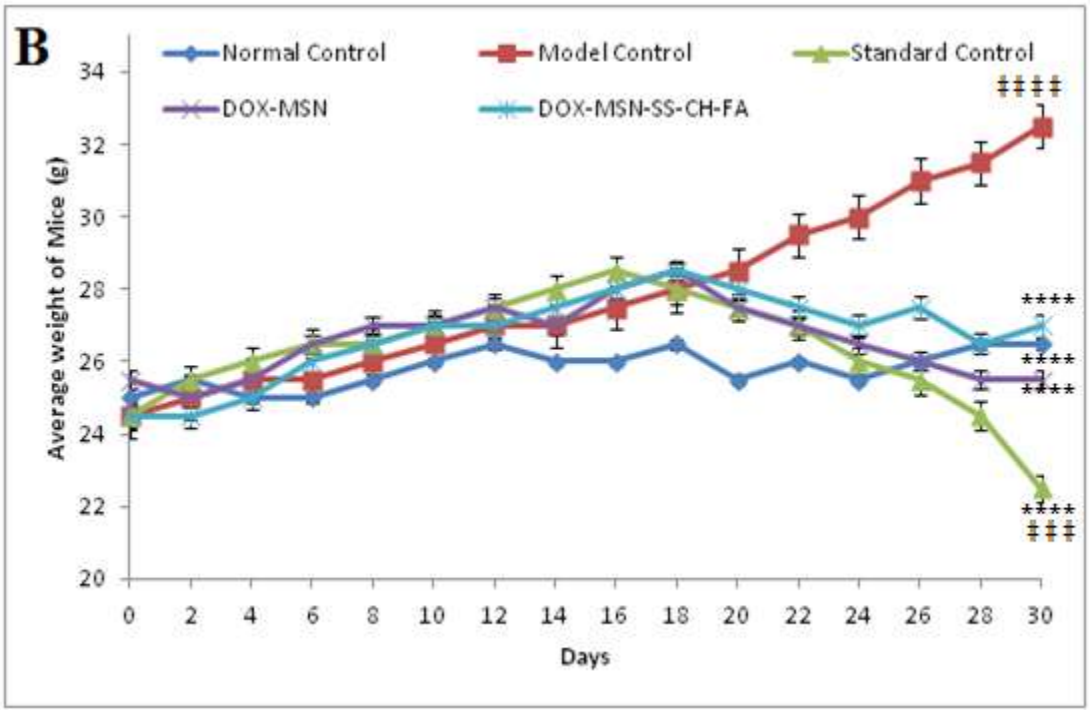
The anti-tumor effectiveness of different formulations was investigated in Balb C mice bearing mammary EAC tumors.

8.8.1 Body weight change:

Figure 8.1 (A, B & C) represent average change in the body weight of the animals as a function of time after inoculation of tumor. Figure 8.1 A depict mice images of control groups. The model control group, injected with EAC, receiving no treatment showed significant weight gain while the standard control group, receiving DOX alone showed significant weight loss.

An initial increase in the weight of all mice (except normal control) was observed after tumor inoculation. Model control group continuously showed increase in the weight till the end of the treatment. This increase in the weight was due to the presence of increased ascitic fluid (observed when the sacrificed mice were dissected). As seen in the figure 8.1 (B & C), the weight change was controlled in all the treatment group mice (except group treated with blank CuO-MSNs) and was significantly different at the end of the treatment as compared to the body weight of model control group ($p<0.0001$). The group receiving DOX alone showed significant reduction in weight and it was considerably different from the weight of normal control group ($p<0.001$). Apart from this, a little decrease in hair around the nose of mice was also observed in mice receiving DOX alone. These weight loss and hair loss are one of the common side effects of chemotherapeutics including DOX. A very little weight loss was observed in mice treated with DOX-MSNs. All the other treatment groups showed noteworthy difference in the body weight ($p<0.0001$) when

compared with the model control group while the body weight was comparable to the normal control group at the end of the treatment.



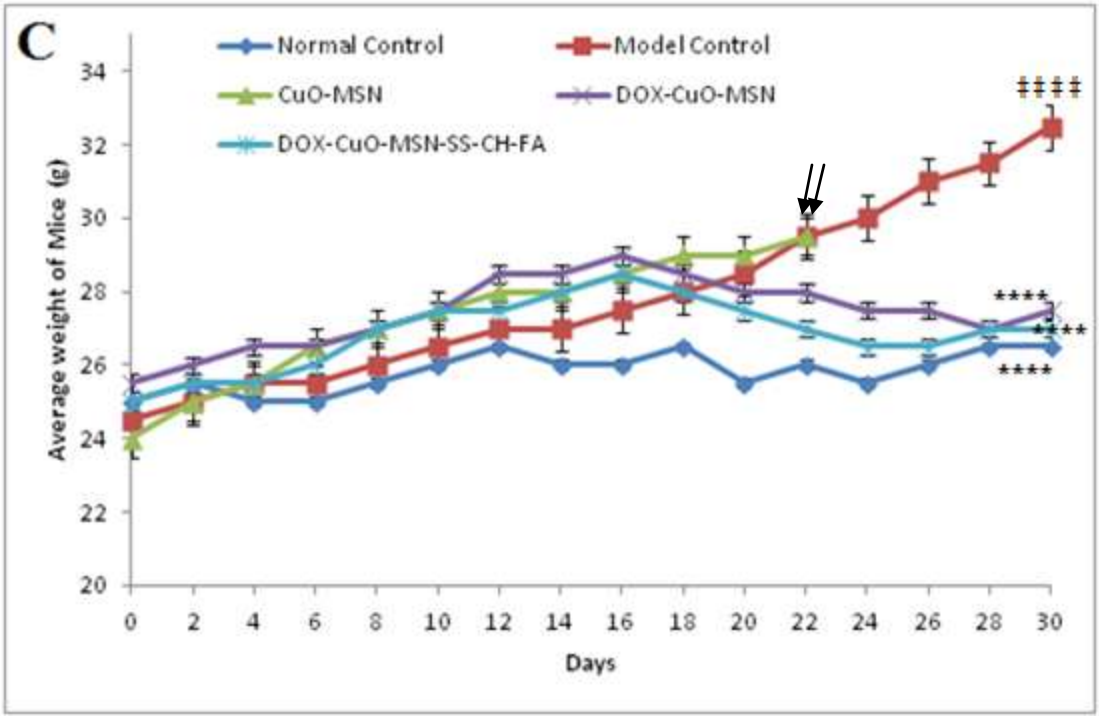


Figure 8.1: Representative image of mice showing weight gain due to EAC and weight loss after Dox treatment (A), Comparison of average body weight change in mice, treated with different MSN formulations (B) and CuO-MSN formulations (C), with control group. **** means $p<0.0001$ when compared with the model control group. ‡‡‡‡ means $p<0.0001$ and ‡‡‡ means $p<0.001$ when compared with the normal control group. Double arrow indicate that all the mice died.

8.8.2 Tumor volume:

Figure 8.2 (A & B) corresponds to change in tumor volume as a function of time. In all the mice administered with EAC, considerable tumor growth was recorded till 15 days. After the initiation of the treatment, a noticeable inhibition of EAC tumor growth was observed in all the treatment groups. In figure 8.2, it is clearly seen that the tumor continued to grow in model control group while it was significantly suppressed ($p<0.0001$ when compared with the model control group) and eventually regressed completely in all treatment groups except CuO-MSN group. A significant difference in the tumor volume measured after 2 doses of DOX-MSN ($p<0.01$) when compared to the standard control group. DOX-MSN-SS-CH-FA showed better tumor inhibition ($p<0.0001$) in comparison to DOX-MSN ($p<0.01$). CuO-MSN alone restricted the growth of tumor locally, but it wasn't capable of treating the tumor completely. This might be due to very

low dose of CuO-MSN. DOX-CuO-MSN showed enhanced efficacy ($p<0.001$) as compared to mice receiving DOX-MSN ($p<0.01$) when compared with the standard control group. DOX-CuO-MSN-SS-CH-FA was found to exhibit the best inhibition ability ($p<0.0001$ when compared with standard control) as compared to other treatment groups This increased efficacy might be due to synergistic effect provided by CuO loaded into the MSNs as CuO-MSN formulations exhibited superior tumor inhibition when compared with MSN formulations.

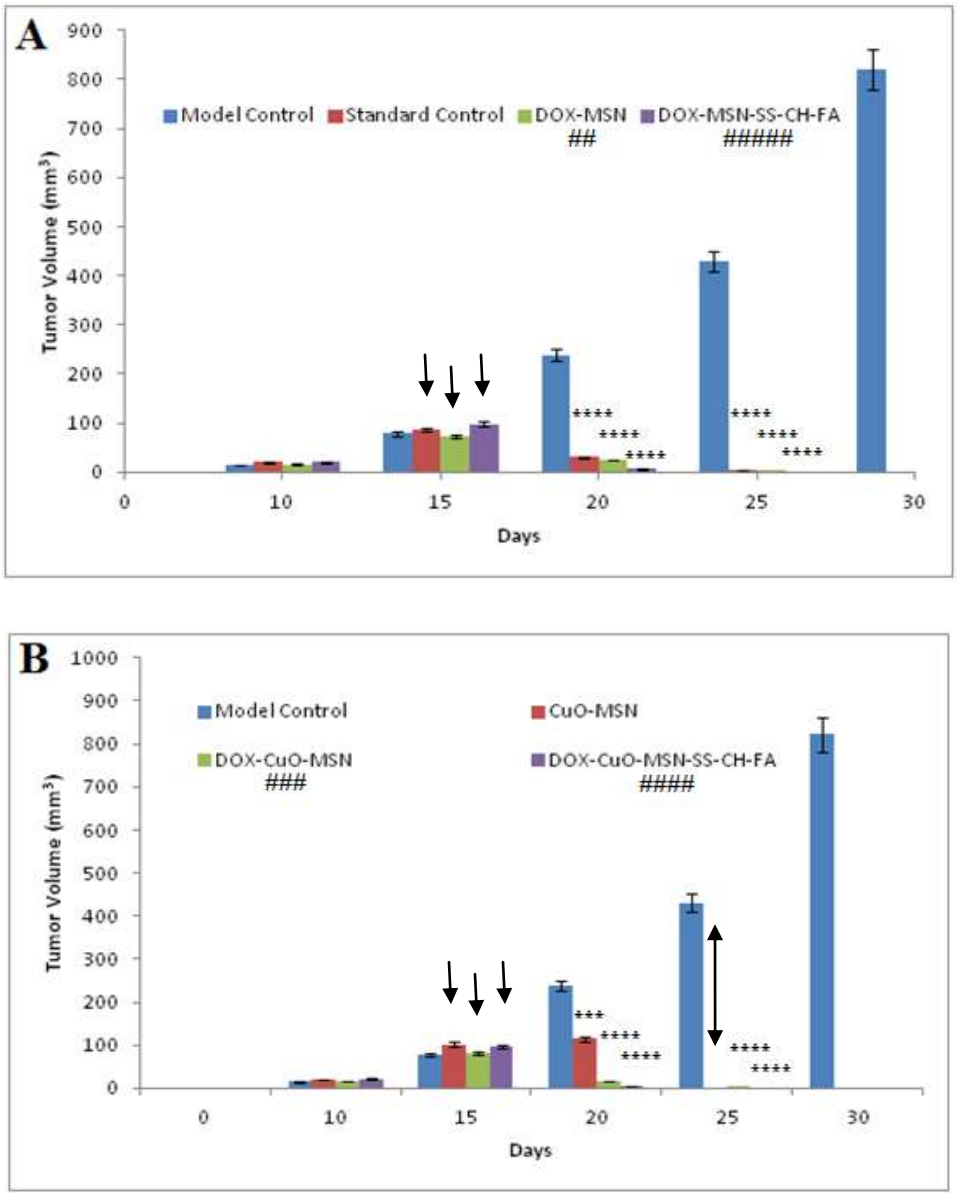


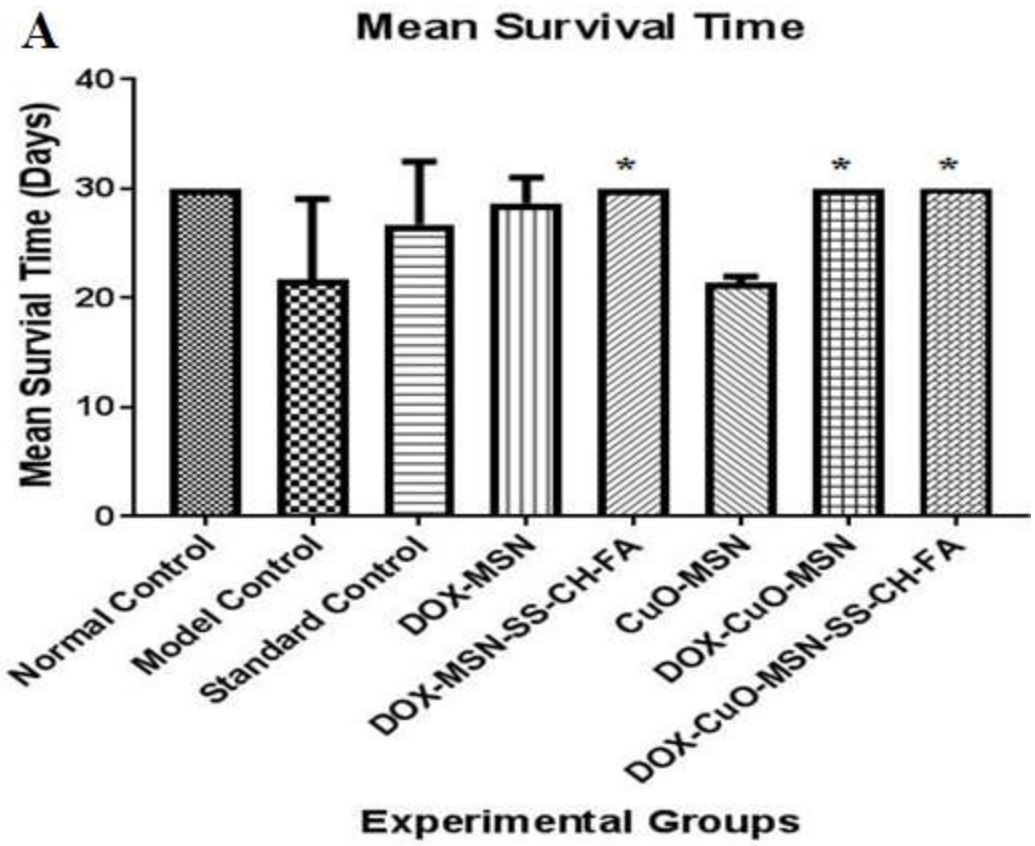
Figure 8.2: Comparison of average change in tumor volume in mice treated with different MSN formulations (A) and CuO-MSN formulations (B), with control group. **** means $p<0.0001$

and *** means $p<0.001$ when compared with the model control group. #### means $p<0.0001$, ### means $p<0.001$ and ## means $p<0.01$ when compared with the standard control group. Single sided arrows indicate the initiation of the treatment while double sided arrow indicate all the mice died.

8.8.3 Survival curve and increased % ILS:

The survival time for different experimental mice inoculated with tumor was observed. After injection of tumor, all the mice were observed up to 30 days and then sacrificed. Figure 8.3 A shows means survival time for all the groups and mice receiving DOX-MSN-SS-CH-FA, DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA showed significant increase in the mean survival time (MST) ($p<0.05$) (100% survival of all mice till the end of the treatment as compared to model control group). The Kaplan Meier survival plot was also plotted for better understanding (Figure 8.3 B). All the mice treated with CuO-MSN alone died leading to the conclusion that, at the given dose CuO-MSN alone was not sufficient to improve survival time. Mice of standard control group and those treated with DOX-MSN showed 66.67% survival at the end of treatment.

Based on the survival data, graph of % increase in the life span was also plotted (Figure 8.3 C). CuO-MSN alone decreased the life span of the treated mice to little extent but it was not significantly different when compared with the model control group. This indicated that the CuO-MSNs might have failed to control metastasis as decrease in the localized tumor volume was observed. Though standard control group showed increase in the life span it wasn't significant as compared to model control group. Though one mouse was died in the groups treated with DOX alone and, DOX-MSN treatment showed increased percent life span (32.31 % ILS as compared to 23.08% ILS of DOX alone). All the treatment groups such as DOX-MSN (32.31 % ILS, $p<0.05$), DOX-MSN-SS-CH-FA (38.46 % ILS, $p<0.01$), DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA ((38.46 % ILS, $p<0.01$ for both) showed considerable increase in the life span as compared to model control group. The mice treated with DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA showed maximum increase in the life span of tumor bearing mice (38.46 %) which was about 1.67 times higher than %ILS observed by DOX alone.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Normal Control
Model Control
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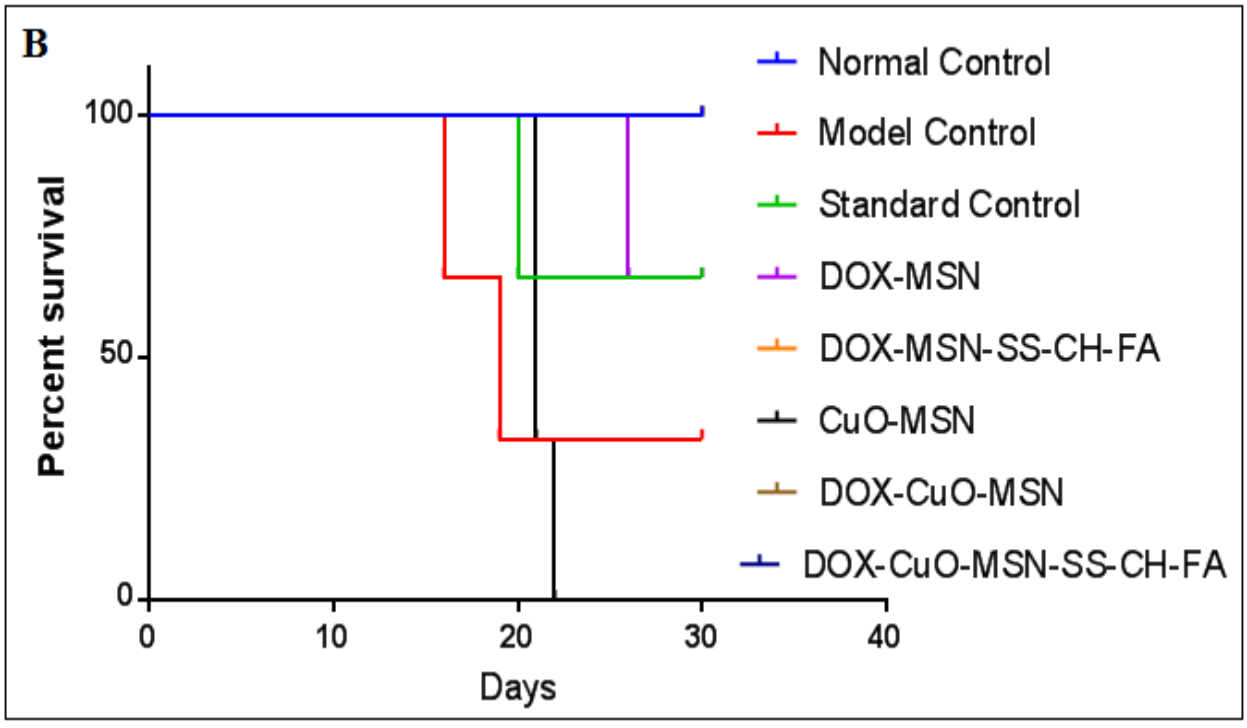
DOX-CuO-MSN
DOX-CuO-MSN-SS-CH-FA
Experimental Groups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DOX-CuO-MSN-SS-CH-FA

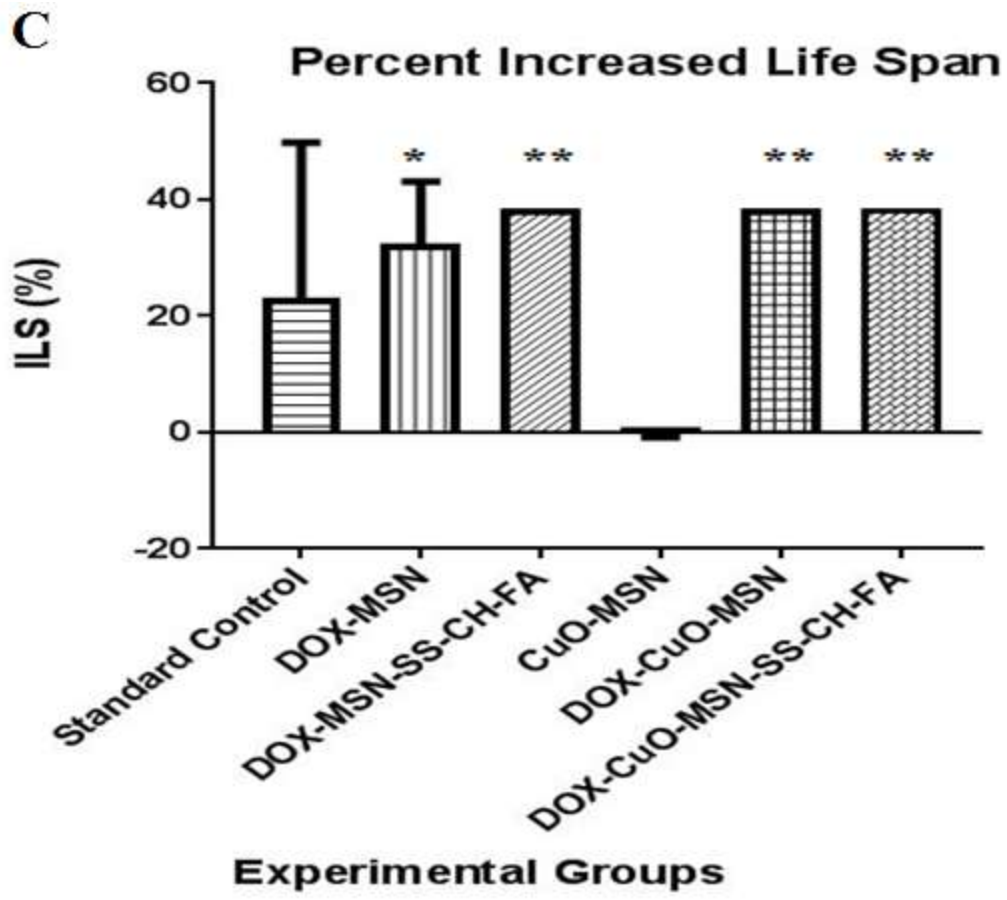


Figure 8.3: Mean survival time of experimental groups (A), Kaplan-Meier survival curve of mice treated with DOX, MSN formulations and CuO-MSN formulations (B), and %ILS of mice treated with DOX, MSN formulations and CuO-MSN formulations compared to control (C). ** means $p<0.01$ and * means $p<0.005$ when compared with the model control group.

8.8.4 Toxicity profiling:

8.8.4.1 Hematological Parameters:

At the end of the treatment, various hematological parameters were checked which are shown in Table 8.4 and 8.5. As compared to normal control, extensive deviation in most of hematological parameters was observed in model control group which might be due to the progression of the disease. Standard control group also showed deviation in various hematological parameters attributed to the adverse effect of DOX.

Table 8.4: Hematological parameters of control group mice.

Parameters	Reference Range	Normal Control	Model Control	Standard Control
WBC * 10^3/µl	5.69-14.84	7.2 ± 0.83	13.8 ± 1.21	4.9 ± 1.47
Lymph* 10^3/µl	0.36-11.56	5.53 ± 0.53	11.26 ± 0.94	3.53 ± 1.20
Mon* 10^3/µl	0.34-1.37	0.39 ± 0.04	0.8 ± 0.08	0.17 ± 0.08
Gran* 10^3/µl	0.75-4.27	1.28 ± 0.24	1.74 ± 0.14	1.2 ± 0.22
Lymph%	55.06-83.82	76.85 ± 11.45	81.59 ± 9.17	65.27 ± 12.03
Mon%	3.75-14.33	5.42 ± 0.38	5.79 ± 0.47	4.23 ± 0.53
Gran%	10.46-33.46	17.8 ± 1.22	12.6 ± 0.91	30.5 ± 8.91
RBC* 10^6/µl	8.16-11.69	9.67 ± 0.48	6.86 ± 0.62	5.36 ± 0.82
HGB g/dl	12.4-18.9	15.58 ± 0.86	10.67 ± 0.54	11.3 ± 0.47
HCT%	43.5-67.0	51.56 ± 3.74	39.33 ± 4.66	35.17 ± 3.46
MCV fl	50.8-64.1	58.13 ± 2.07	50.3 ± 5.10	45.6 ± 3.17
MCH pg	13.0-17.6	14.23 ± 0.67	13.83 ± 0.47	13.13 ± 0.34
MCHC g/dl	23.9-33.1	29.27 ± 0.93	24.97 ± 0.33	30.2 ± 0.22

RDW %	16.9-23.5	18.17 ± 1.10	16.87 ± 1.70	14.7 ± 3.60
PLT* 10^3/µl	476-1611	526.67 ± 44.83	426.87 ± 40.80	251 ± 30.70
MPV fl	4.6-5.8	5.13 ± 0.57	4.77 ± 0.30	6.33 ± 1.20
PDW %	-	14.33 ± 0.87	15.67 ± 1.10	15.9 ± 0.90
PCT %	-	0.31 ± 0.04	0.19 ± 0.03	0.16 ± 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.

Table 8.5 represents the hematological parameters of different treatment group mice. All the mice of CuO-MSN treatment group died before completion of experiment and hence, hematological and serological parameters of that group were not measured. Almost all the parameters were found normal in the mice treated with DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA while some of the parameters such as monocytes, hemoglobin, mean corpuscular volume (MCV) were observed little low in case of mice treated with DOX-MSN and DOX-CuO-MSN which might be due to premature release of DOX into the blood.

Table 8.5: Hematological parameters of treatment group mice.

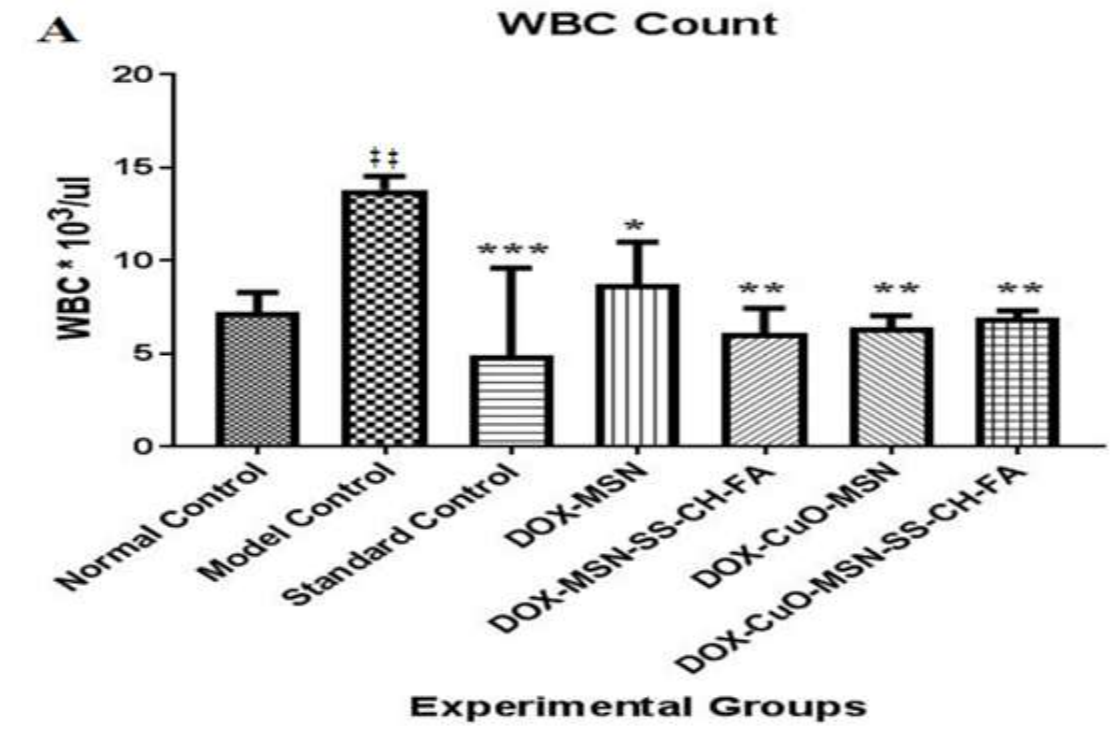
Parameters	**Reference Range**	**DOX-MSN**	**DOX-MSN-SS-CH-FA**	**DOX-CuO-MSN**	**DOX-CuO-MSN-SS-CH-FA**
WBC * 10^3/µl	5.69-14.84	8.73 ±	6.1 ± 0.64	6.4 ± 0.91	6.9 ± 0.72

		1.03			
Lymph* 10^3/µl	0.36-11.56	6.47 ± 0.67	4.33 ± 0.44	4.17 ± 0.62	5.17 ± 0.47
Mon* 10^3/µl	0.34-1.37	0.3 ± 0.10	0.33 ± 0.04	0.31 ± 0.03	0.35 ± 0.05
Gran* 10^3/µl	0.75-4.27	1.97 ± 0.07	1.44 ± 0.12	1.92 ± 0.25	1.38 ± 0.18
Lymph%	55.06-83.82	74.4 ± 6.43	70.98 ± 8.41	65.15 ± 6.52	74.93 ± 8.40
Mon%	3.75-14.33	3.6 ± 0.91	5.41 ± 0.54	4.83 ± 0.63	5.07 ± 0.48
Gran%	10.46-33.46	22 ± 2.11	24.63 ± 2.69	30 ± 2.42	20 ± 1.77
RBC* 10^6/µl	8.16-11.69	8.34 ± 0.33	8.97 ± 0.36	8.67 ± 0.38	9.43 ± 0.41
HGB g/dl	12.4-18.9	11.9 ± 0.56	14.57 ± 0.40	13.37 ± 0.34	15.67 ± 0.71
HCT%	43.5-67.0	36.7 ± 0.48	48.83 ± 2.81	41.56 ± 2.73	52.83 ± 2.56
MCV fl	50.8-64.1	44.1 ± 2.27	49.33 ± 2.46	48.13 ± 2.61	56.6 ± 3.15
MCH pg	13.0-17.6	14.23 ± 0.53	13.93 ± 0.75	13.33 ± 0.67	14.67 ± 0.91
MCHC g/dl	23.9-33.1	32.37 ± 0.37	32.37 ± 1.43	30.1 ± 1.63	30.13 ± 0.82

RDW %	16.9-23.5	13.63 ± 0.37	14 ± 0.93	15.43 ± 1.10	17.87 ± 1.22
PLT* 10^3/µl	476-1611	494.67 ± 74.67	520 ± 40.00	510.13 ± 51.66	530.6 ± 47.00
MPV fl	4.6-5.8	5.17 ± 0.57	5.567 ± 0.51	4.83 ± 0.63	5.2 ± 0.53
PDW %	-	14.7 ± 0.37	15.1 ± 0.98	14.33 ± 1.23	15.2 ± 0.81
PCT %	-	0.25 ± 0.05	0.29 ± 0.04	0.27 ± 0.03	0.32 ± 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.

Various blood parameters such as white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB) and platelets were compared graphically and showed in figure 8.4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Normal Control
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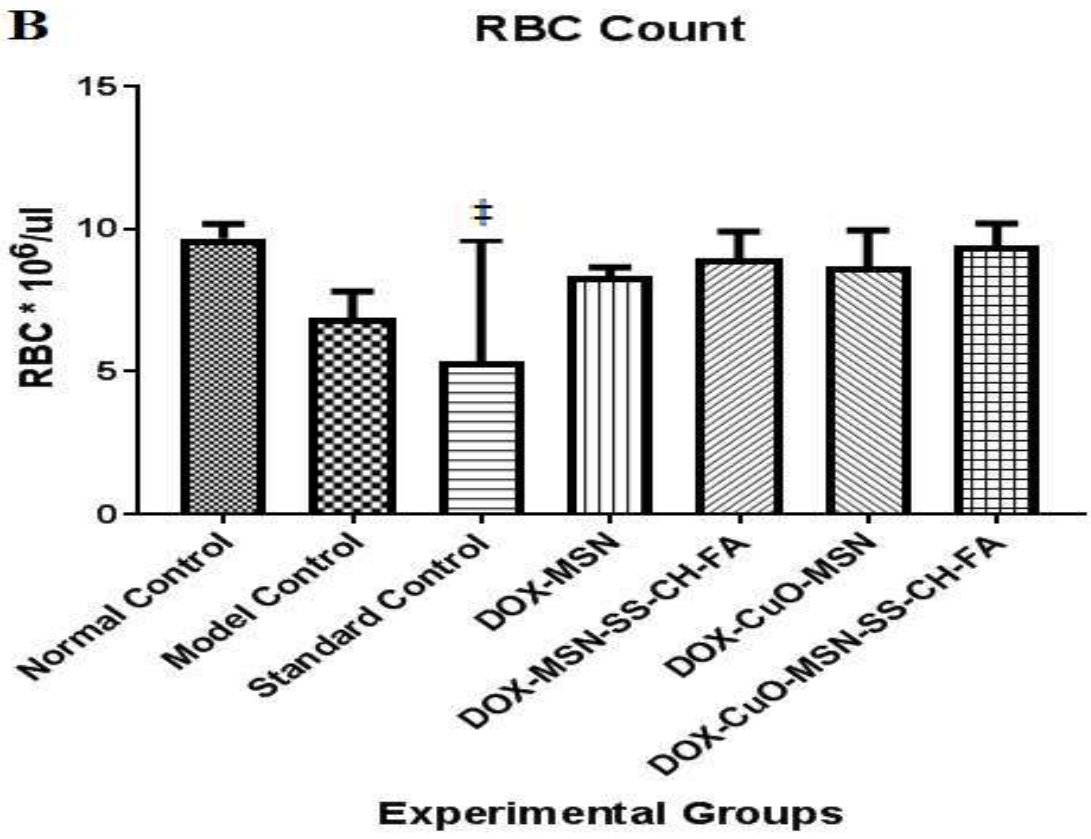
DOX-CuO-MSN-SS-CH-FA
Experimental Groups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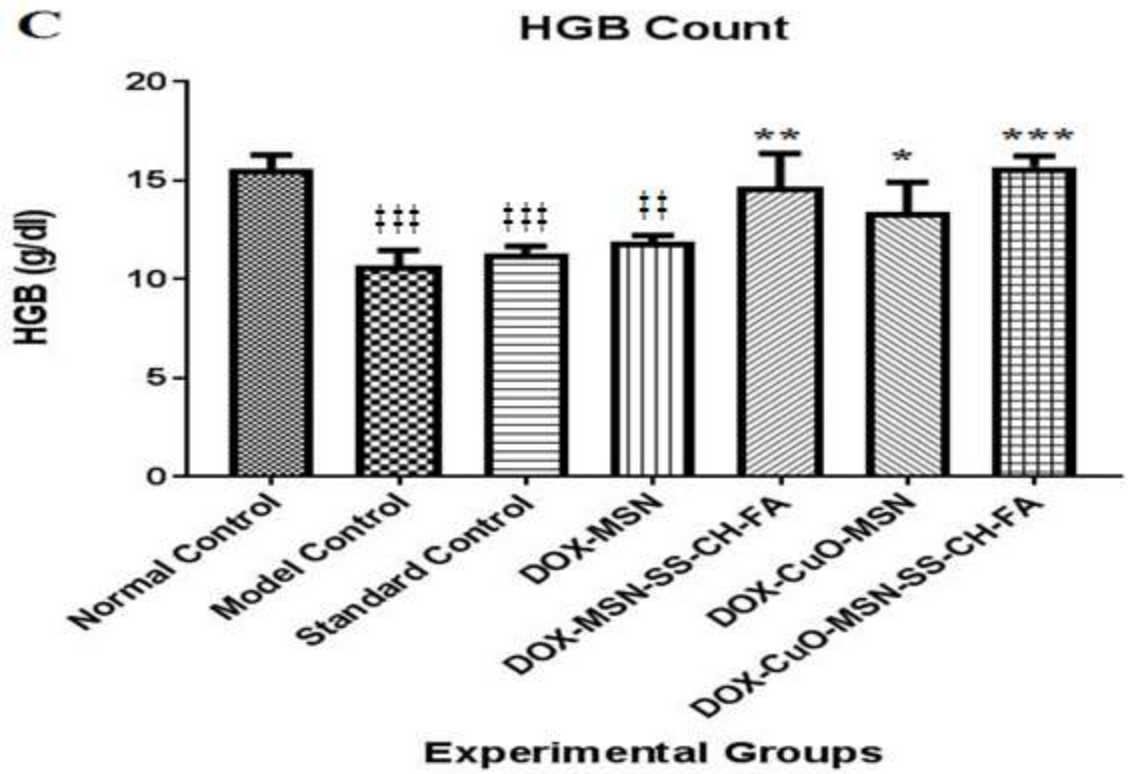
Experimental Groups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Normal Control
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DOX-CuO-MSN-SS-CH-FA
Experimental Groups

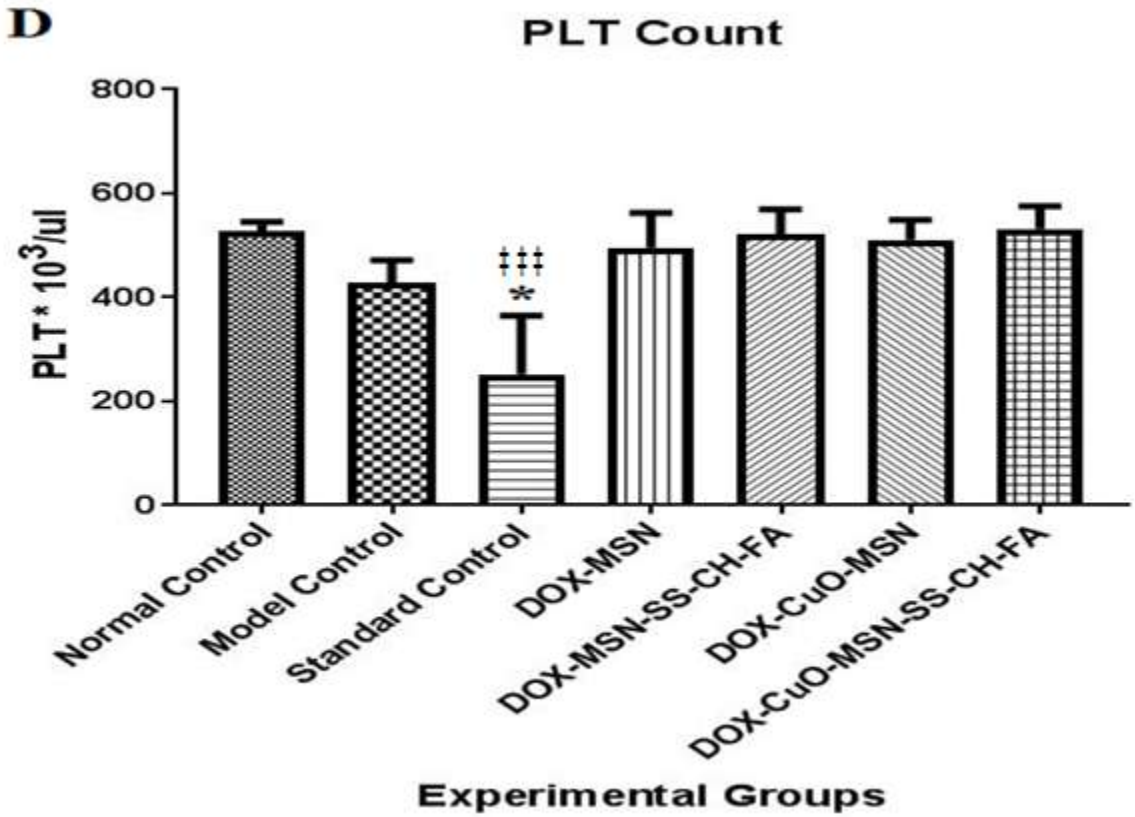

D
PLT Count
PLT * 10^3/ul
800
600
400
200
0
‡‡‡
*
Normal Control
Model Control
Standard Control
DOX-MSN
DOX-MSN-SS-CH-FA
DOX-CuO-MSN
DOX-CuO-MSN-SS-CH-FA
Experimental Groups

Figure 8.4: Comparison of hematological parameters such as WBCs (A), RBCs (B), HGB (C) and PLT (D) of different treatment group mice and control group mice. *** means $p<0.001$, ** means $p<0.01$ and * means $p<0.05$ when compared with the model control group. ‡‡‡ means $p<0.001$, ‡‡ means $p<0.01$ and ‡ means $p<0.05$ when compared with the normal control group.

As shown in figure 8.4 A, all the groups showed significant difference in WBC as compared to model control group ($p<0.05$). About 1.92 fold higher WBC count was recorded in model control group as compared to normal control. DOX treatment group showed marked decrease (about 31% compared to normal control) in the WBCs. No significant difference in RBCs was observed when compared with model control group but RBCs were found to be decreased in DOX treated group ($p<0.05$) as compared to normal control group. Apart from this, reduction in HGB was also seen ($p<0.001$ compared to normal control) in mice treated with DOX alone. Furthermore, mice that received DOX alone showed drop in platelet counts. Myelosuppression and anaemia are the major problems during cancer chemotherapy which can result in reduction of above mentioned hematological parameters. DOX toxicity may cause anaemia by altering the activity of hematopoietic tissues, impaired erythropoiesis, and/or defective iron metabolism.[9] None of the treatment groups demonstrated considerable difference in RBCs and PLT when compared with the model control group or normal control group. Treatment groups DOX-MSN-SS-CH-FA ($p<0.01$), DOX-CuO-MSN ($p<0.05$) and DOX-CuO-MSN-SS-CH-FA ($p<0.001$) showed considerable increase in HGB compared to model control group. Mice treated with DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA showed almost all the hematological parameters comparable to normal control group. DOX-MSN and DOX-CuO-MSN also showed improved hematological parameters as compared to free drug. It clearly proved that use of MSNs and CuO-MSNs as carrier for DOX can significantly reduce the hematological adverse effects associated with DOX treatment.

8.8.4.2 Hepatotoxicity:

With a view to measure liver functionality, different liver enzymes such as AST, ALT and GGT were measured. The GGT was found 0 in all the experimental groups. So there wasn't any change associated with GGT.

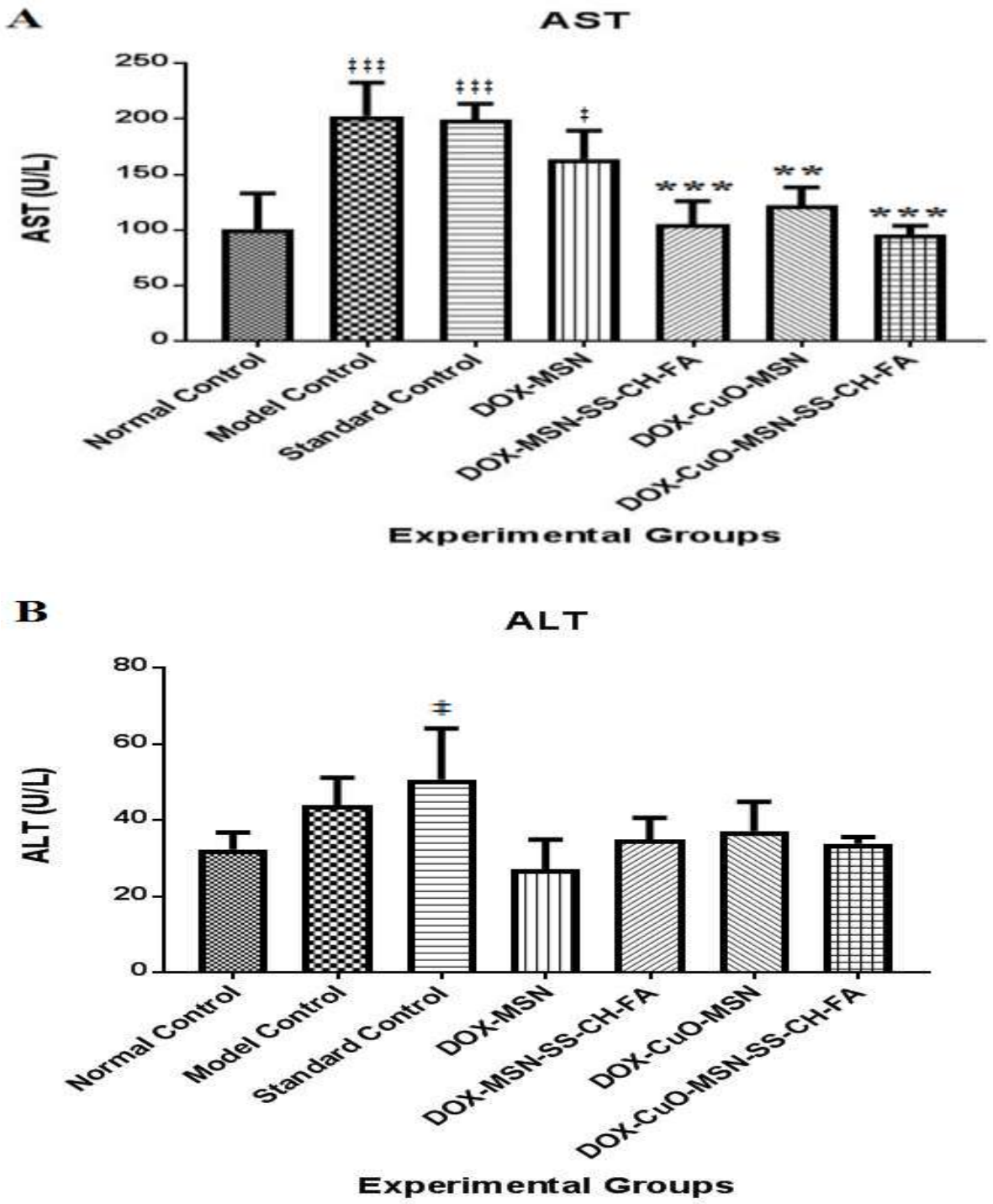


Figure 8.5: Comparison of hepatic markers AST (A) and ALT (B) in the serum of different experimental groups. *** means $p<0.001$, ** means $p<0.01$ when compared with the model control group. ‡‡‡ means $p<0.001$, and ‡ means $p<0.05$ when compared with the normal control group.

Model control group showed marked increase in both AST ($p<0.001$) and ALT as compared to normal group (Figure 8.5). So there is a chance that the tumor was metastasized to liver, due to which hepatic function was impaired. Mice treated with DOX alone ($p<0.001$) and DOX-MSN ($p<0.05$) showed elevation in AST as compared to normal control group. DOX group also showed little increase ($p<0.05$) in ALT as compared to normal control group. So this increase might be indicative of hepatic toxicity associated with DOX as model control did not show increased ALT when compared with normal control. Mice receiving DOX-MSN-SS-CH-FA ($p<0.01$), DOX-CuO-MSN ($p<0.05$) and DOX-CuO-MSN-SS-CH-FA ($p<0.001$) exhibited significant reduction in AST as compared to model control group and it was comparable to normal control group. However, no significant difference was observed in ALT between treatment group mice and normal as well as model control group mice. As no significant difference in AST and ALT level in mice receiving DOX-MSN-SS-CH-FA, DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA was observed compared to normal control, adequate functioning of liver was proven in mice treated with these formulations.

8.8.4.3 Renal toxicity:

BUN and serum creatinine are generally used to measure the kidney function. Figure 8.6 shows comparison of BUN and creatinine found in the serum of different experimental animals. As seen in figure 8.6, both creatinine and BUN were markedly elevated in model control group ($p<0.05$ for bothas compared to normal control group) clearly indicating damaged renal functionality. Mice receiving DOX-MSN ($p<0.05$), DOX-MSN-SS-CH-FA ($p<0.01$), DOX-CuO-MSN ($p<0.01$) and DOX-CuO-MSN-SS-CH-FA showed significant decrease in the creatinine when compared to model control group. Mice treated with DOX-MSN-SS-CH-FA ($p<0.05$) and DOX-CuO-MSN-SS-CH-FA ($p<0.01$) in comparison to model control group. These groups did not show any significant difference when compared with model control group proving the adequate functioning of kidneys.

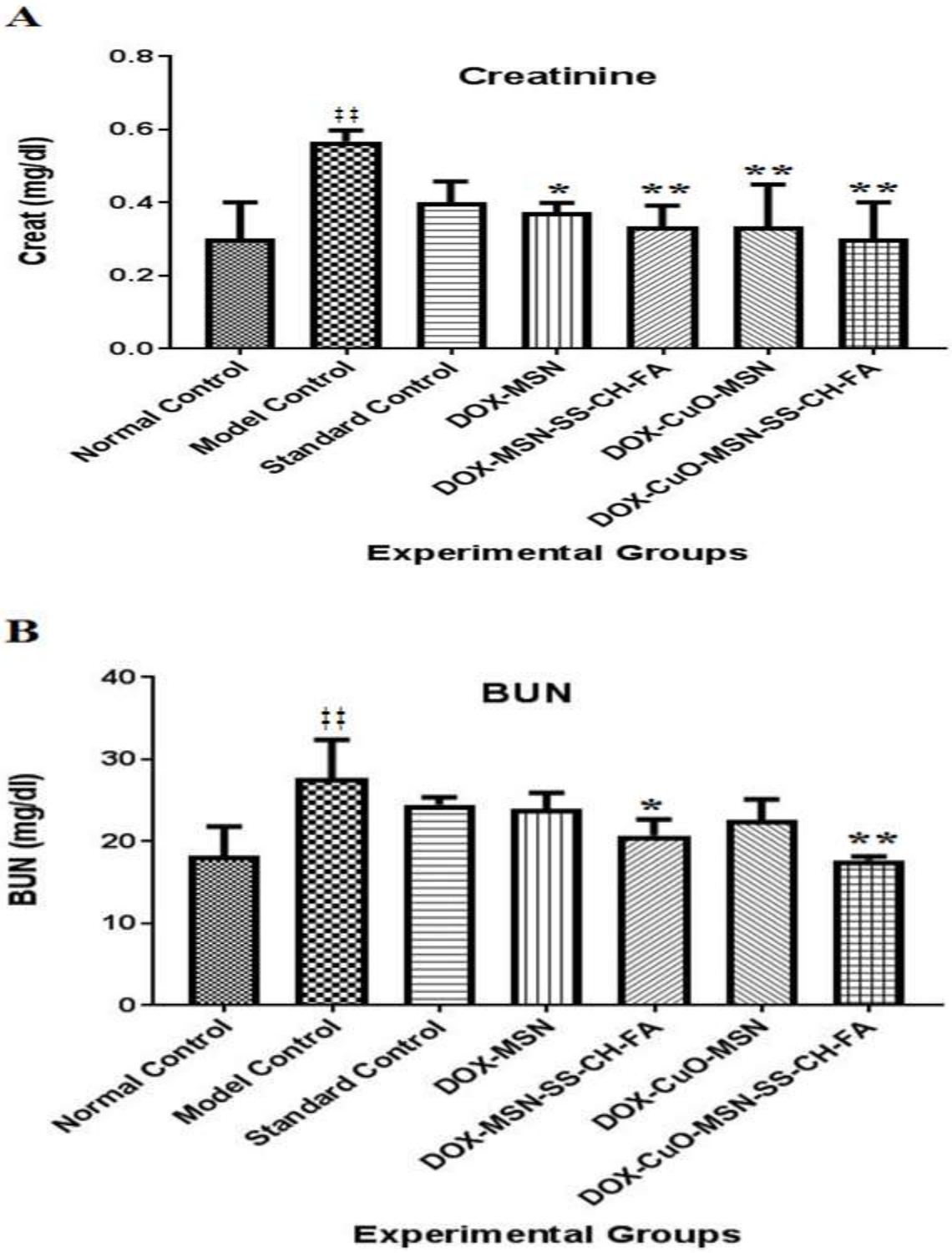


Figure 8.6: Comparison of renal markers creatinine (A) and BUN (B) in the serum of different experimental groups. ** means $p<0.01$ and * means $p<0.05$ when compared with the model control group. ‡‡ means $p<0.01$, and ‡ means $p<0.05$ when compared with the normal control group.

8.8.4.4 Cardiotoxicity:

DOX induced cardiotoxicity is well known and a major side effect associated with DOX treatment. CK-MB is an enzyme which is used to evaluate the occurrence and extent of myocyte injury. It is regarded as a gold standard, by World health organization (WHO), as indicative of myocardial damage and is widely used in research involving cardiotoxicity.[10]

Levels of CK-MB in diverse experimental groups is shown in figure 8.7. As the nanoparticles are hypothesized to target breast cancer cells exclusively and reduce the toxicities associated with free DOX, estimation of CK-MB is a crucial parameter for measurement of cardiac toxicity. As seen in figure 8.7, CK-MB levels were noticeably elevated in standard control group receiving free DOX ($p<0.0001$). Mice receiving different nanoparticles showed significant reduction in the CK-MB level which clearly proved considerable reduction in the DOX induced cardiotoxicity.

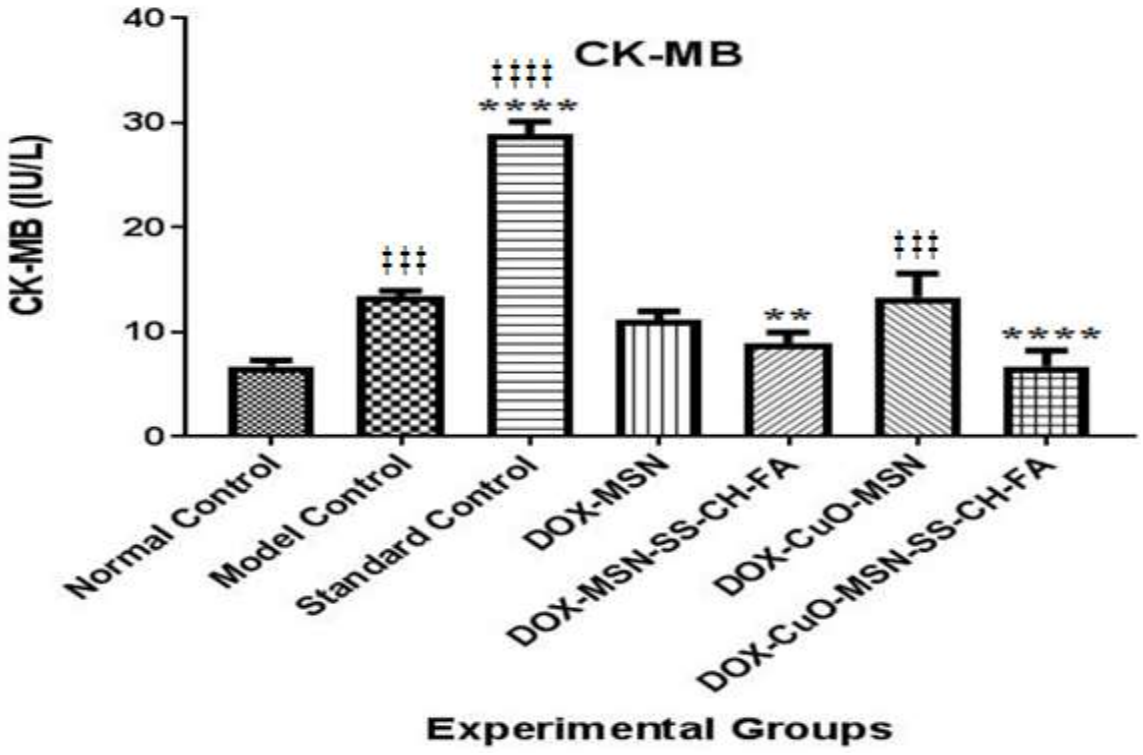


Figure 8.7: Comparison of CK-MB as a cardiac marker in the serum of different experimental groups. **** means $p<0.0001$ and ** means $p<0.01$ when compared with

the model control group. ‡‡‡‡ means p<0.0001, and ‡‡‡ means p<0.001 when compared with the normal control group.

Apart from these, total protein (T. protein) content was also measured in the serum of all the mice. As seen in figure 8.8, decrease was observed in the T. protein content in mice of model control group (p<0.001) and mice treated with DOX (p<0.0001)when compared with the normal control group. In all the other treatment groups, the T.protein content was comparable to the normal control group and significantly elevated with p<0.0001 when compared to model control group.

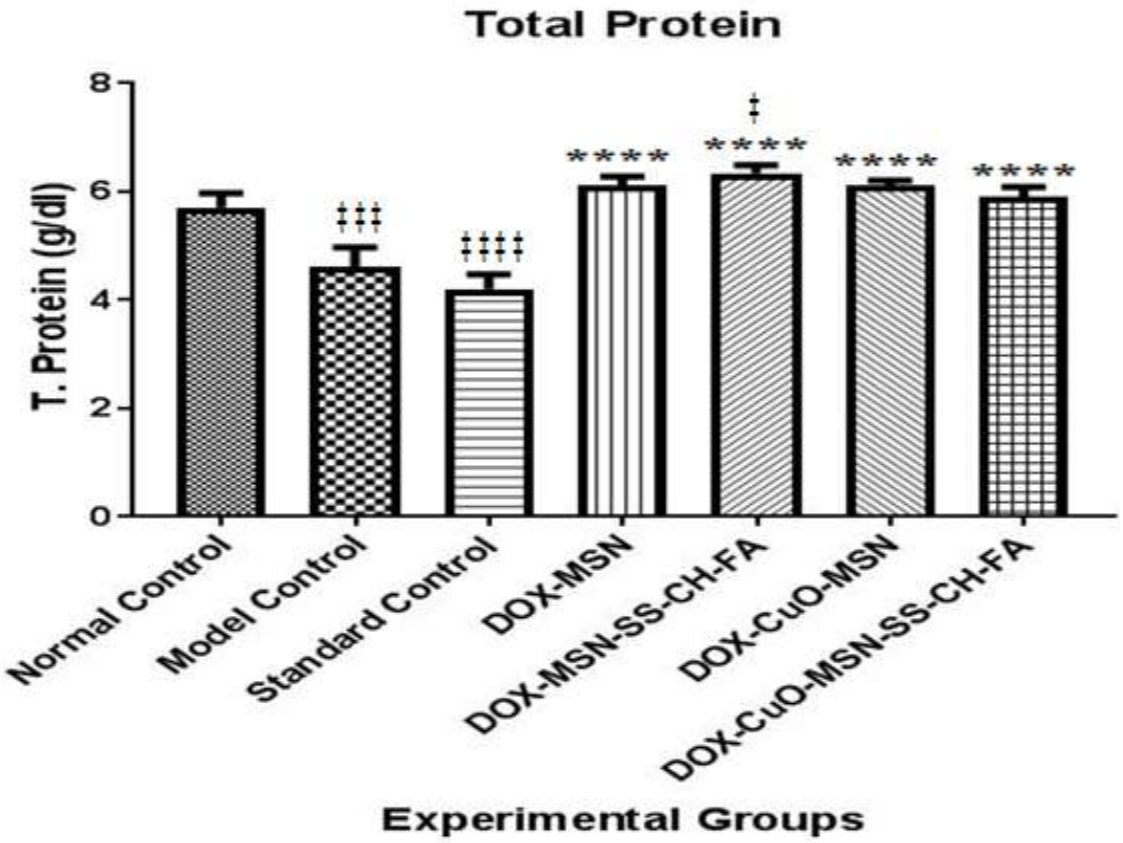
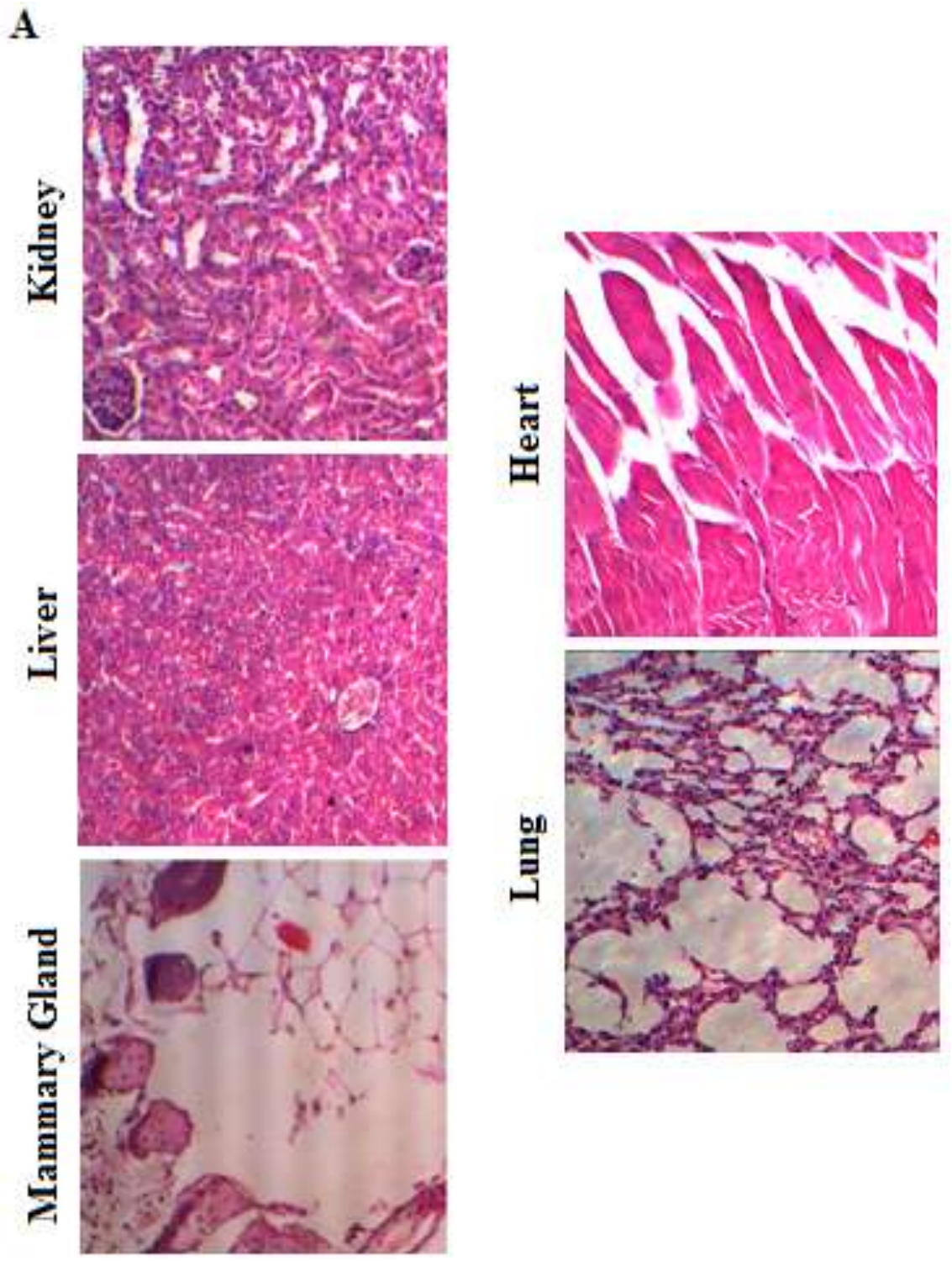
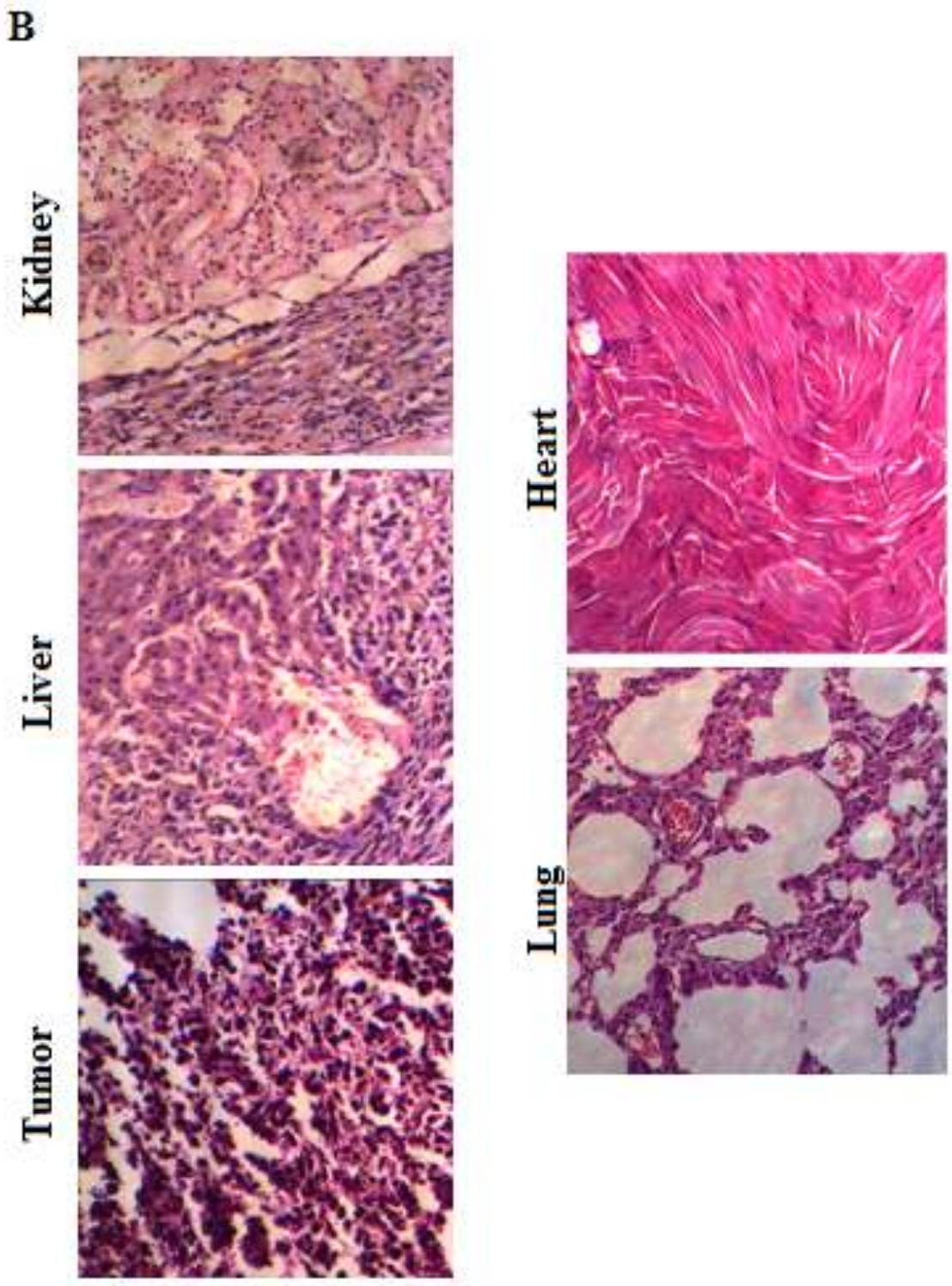


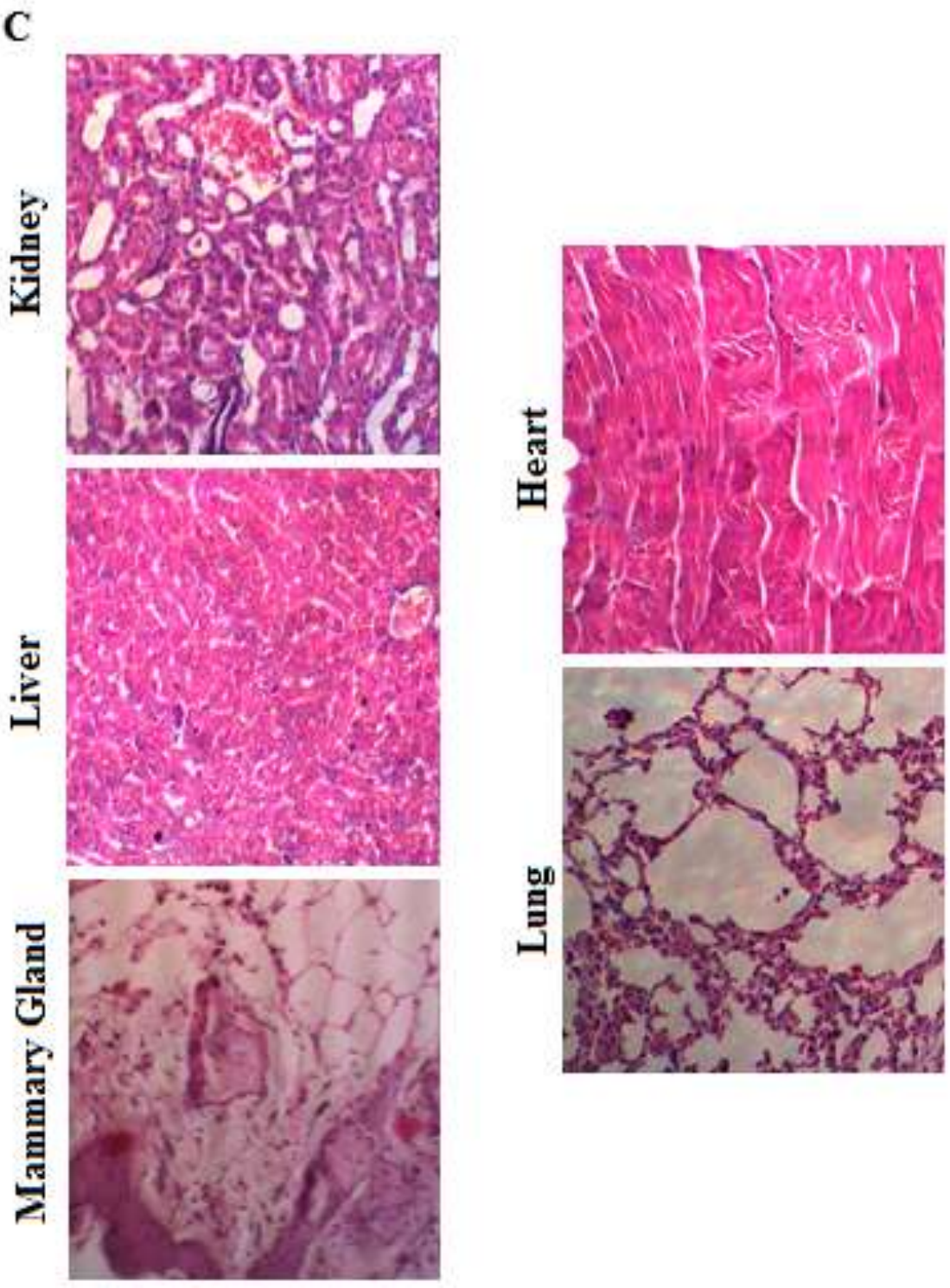
Figure 8.8: Comparison of total protein in the serum of different experimental groups. ** means p<0.01 and * means p<0.05 when compared with the model control group. ‡‡ means p<0.01, and ‡ means p<0.01 when compared with the model control group.

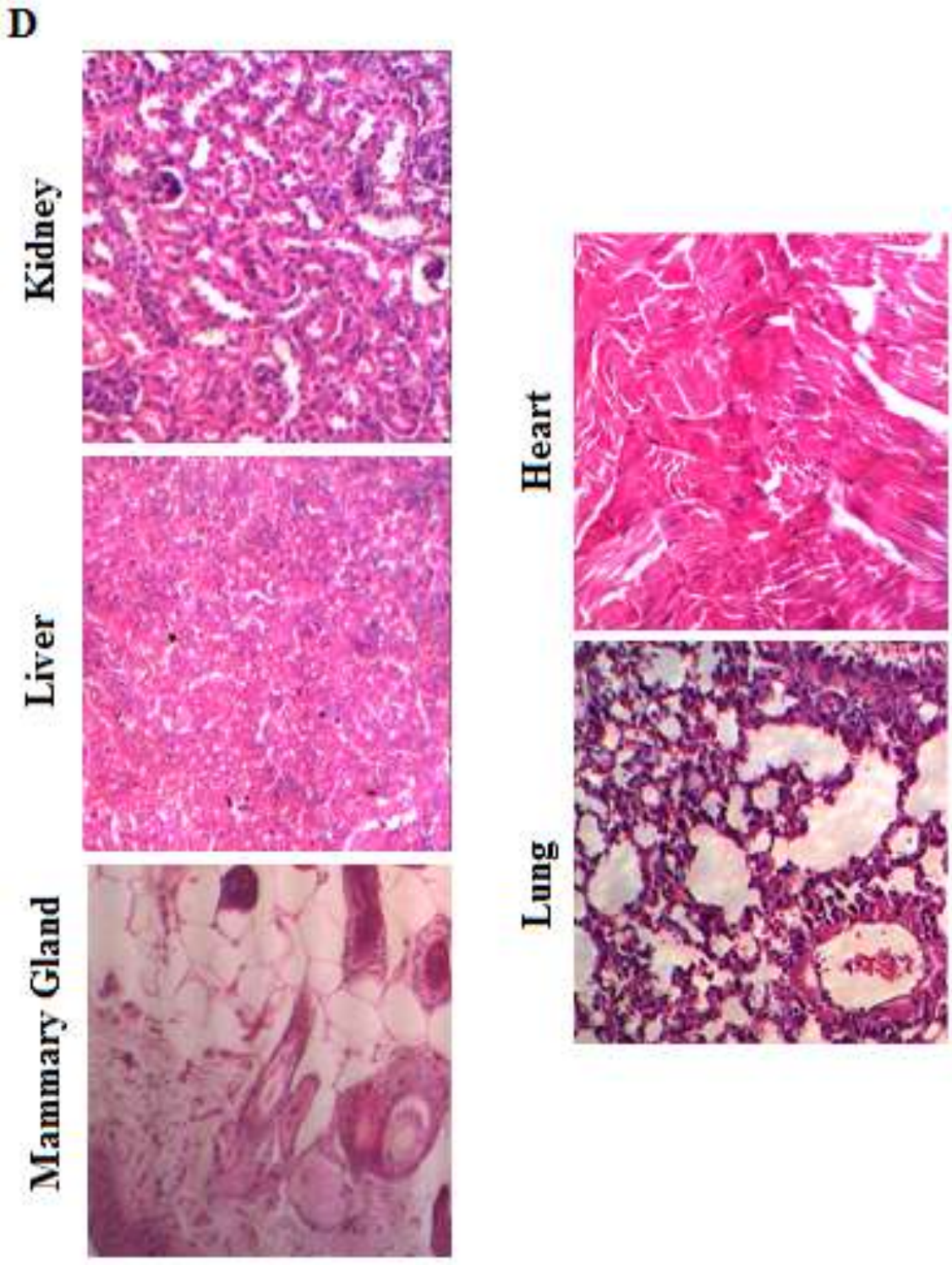
8.8.5 Histopathological Examination:

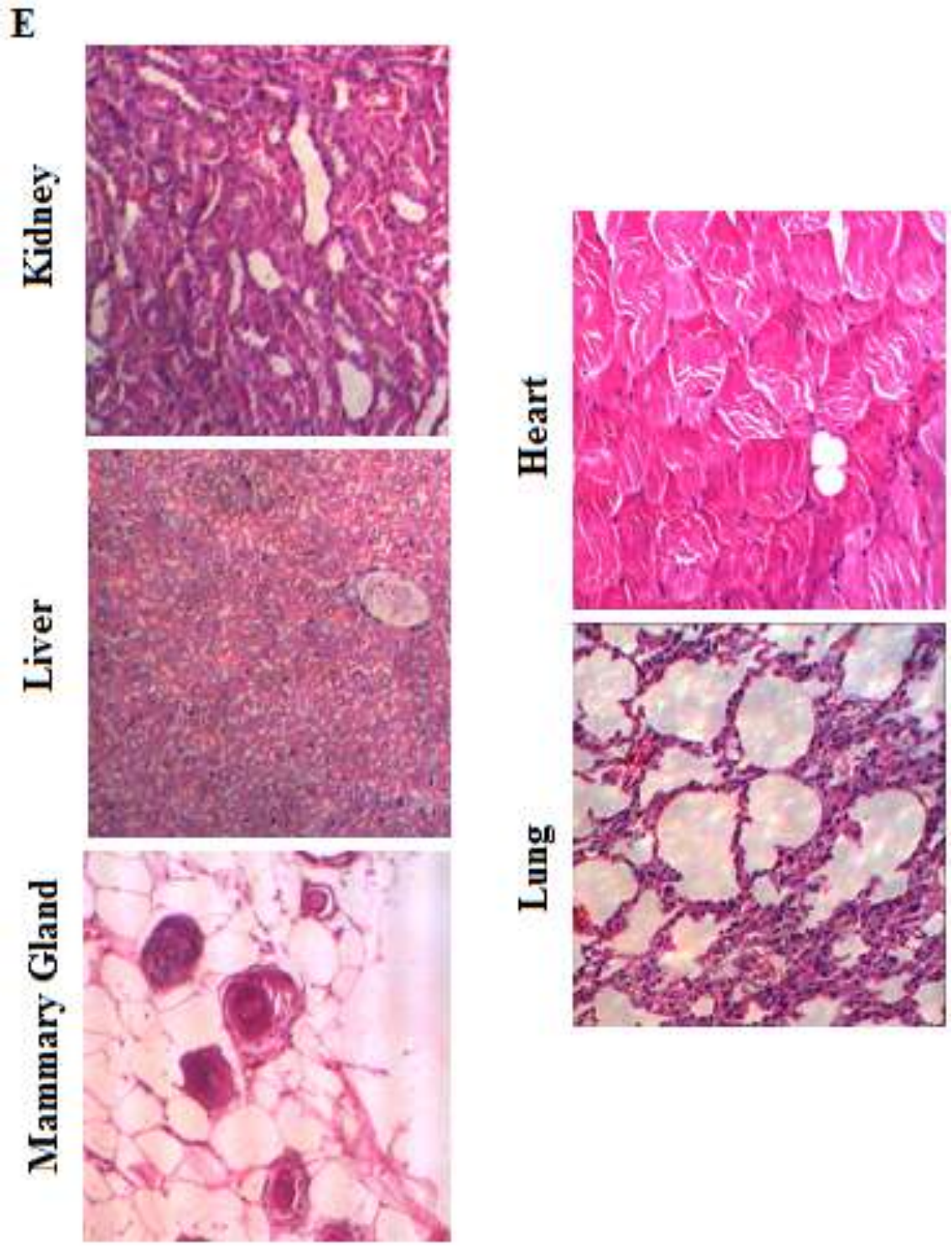
At the end of the experiment all the mice were sacrificed and histopathological examination of mammary gland/tumor, liver, lungs, kidneys and heart was performed. In a gross diagnosis an enlarged liver was observed in model control group and standard control group. Figure 8.9 correspond to histapathological examination of different tissues of all groups. Heart and lungs of all the treatment groups were found normal without any abnormality. The presence of tumor in mammary gland of model control group and CuO-MSN treatment group was observed. It can be clearly seen that the tumor was metastasized to liver in both model control group as well as in CuO-MSN group, while tumor was found to invade the capsule of kidney in model control group. In CuO-MSN group, phagocytes attacking the incoming tumor were visualized. In all the other treatment groups, the tumor was completely regressed and no abnormality was detected in the mammary gland. Similarly, histopathology of all other organs of mice treated with DOX, DOX-MSN, DOX-MSN-SS-CH-FA, DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA was also found comparable with the normal control group without any signs of abnormality. This clearly shows that the treatment not only treated the tumor but also prevented the metastasis also.

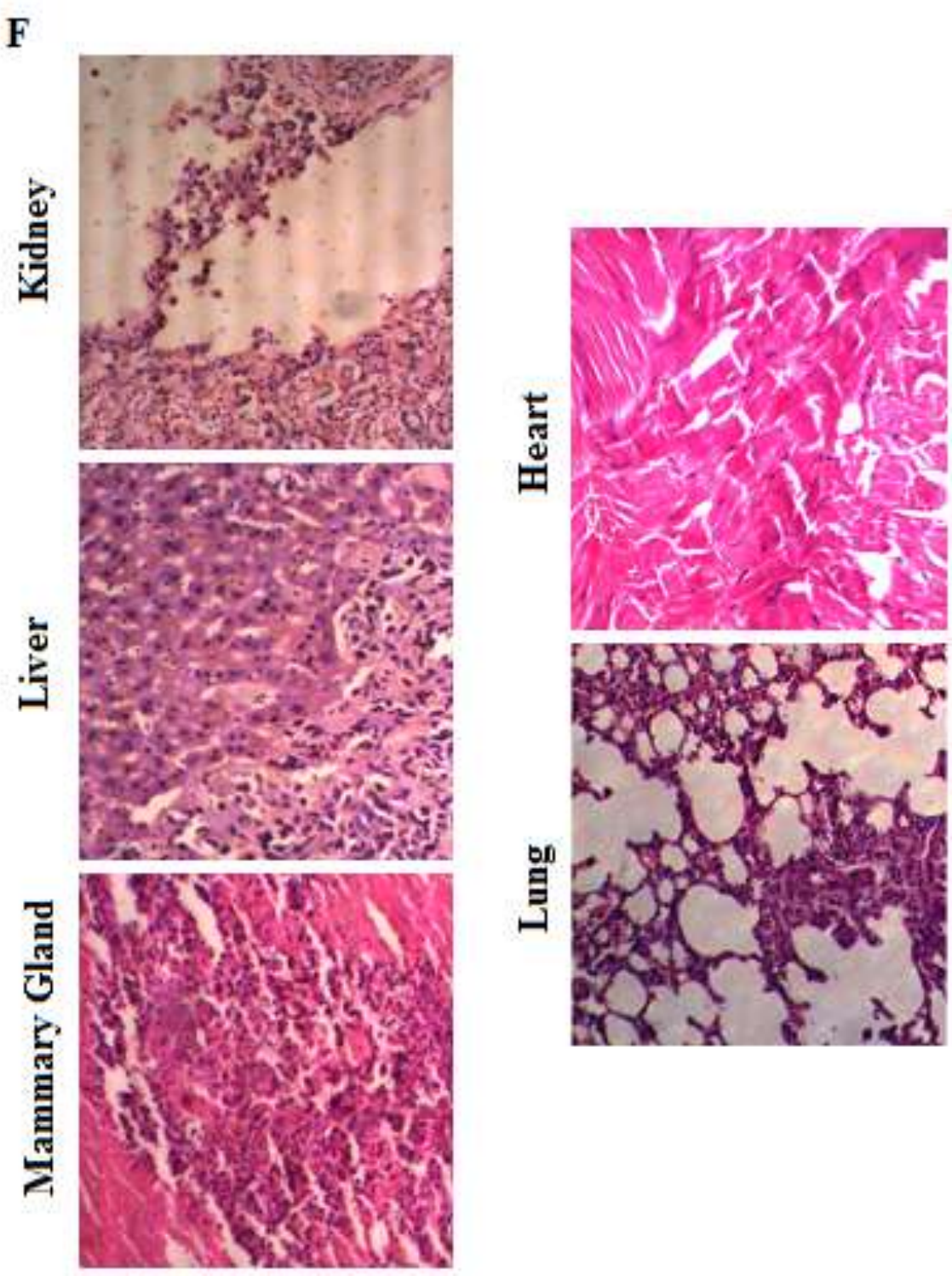

A
Kidney
Liver
Mammary Gland
Heart
Lung


B
Kidney
Liver
Tumor
Heart
Lung


C
Kidney
Liver
Mammary Gland
Heart
Lung


D
Kidney
Liver
Mammary Gland
Heart
Lung


E
Kidney
Liver
Mammary Gland
Heart
Lung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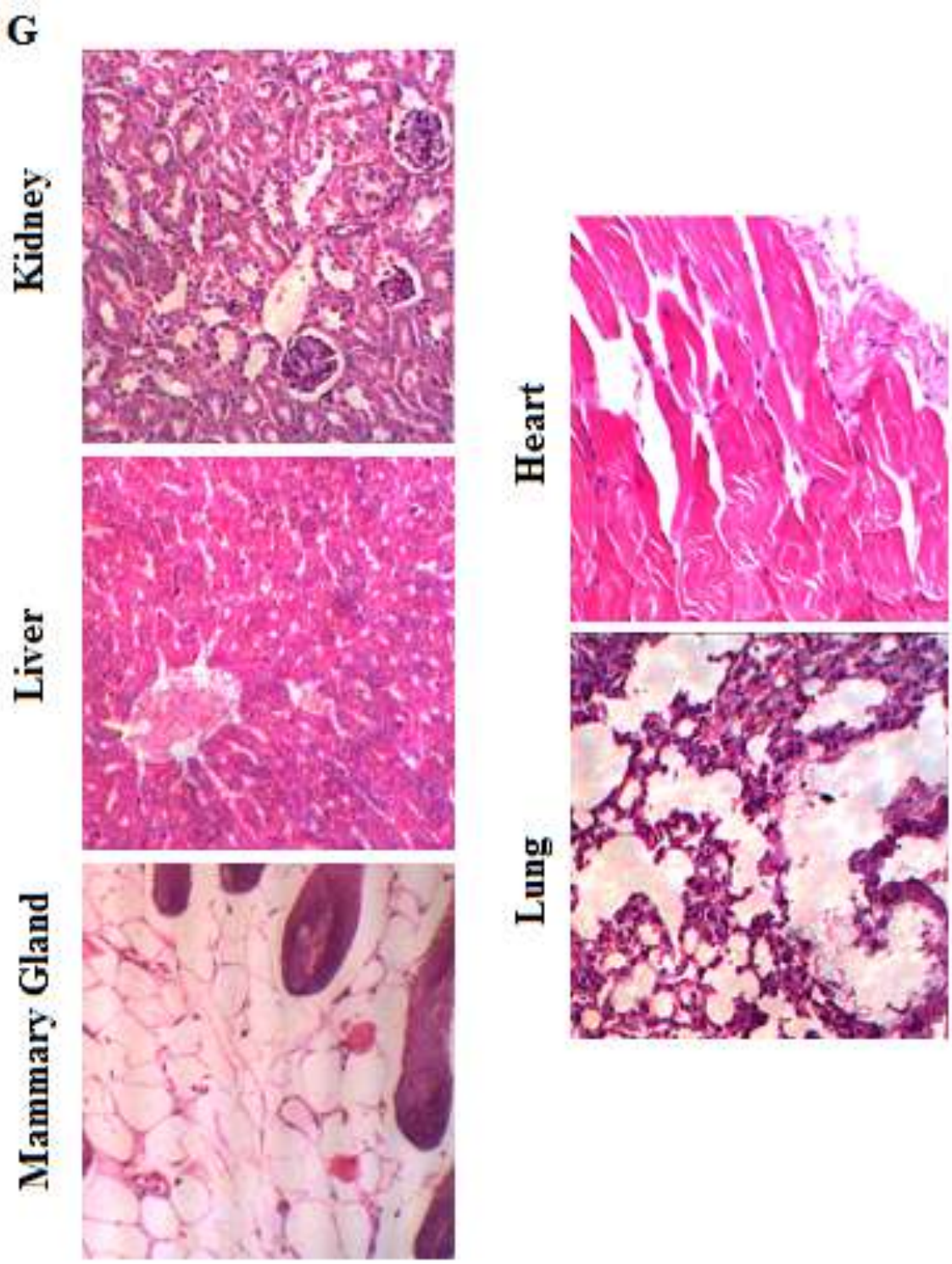
G
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Heart
Lung

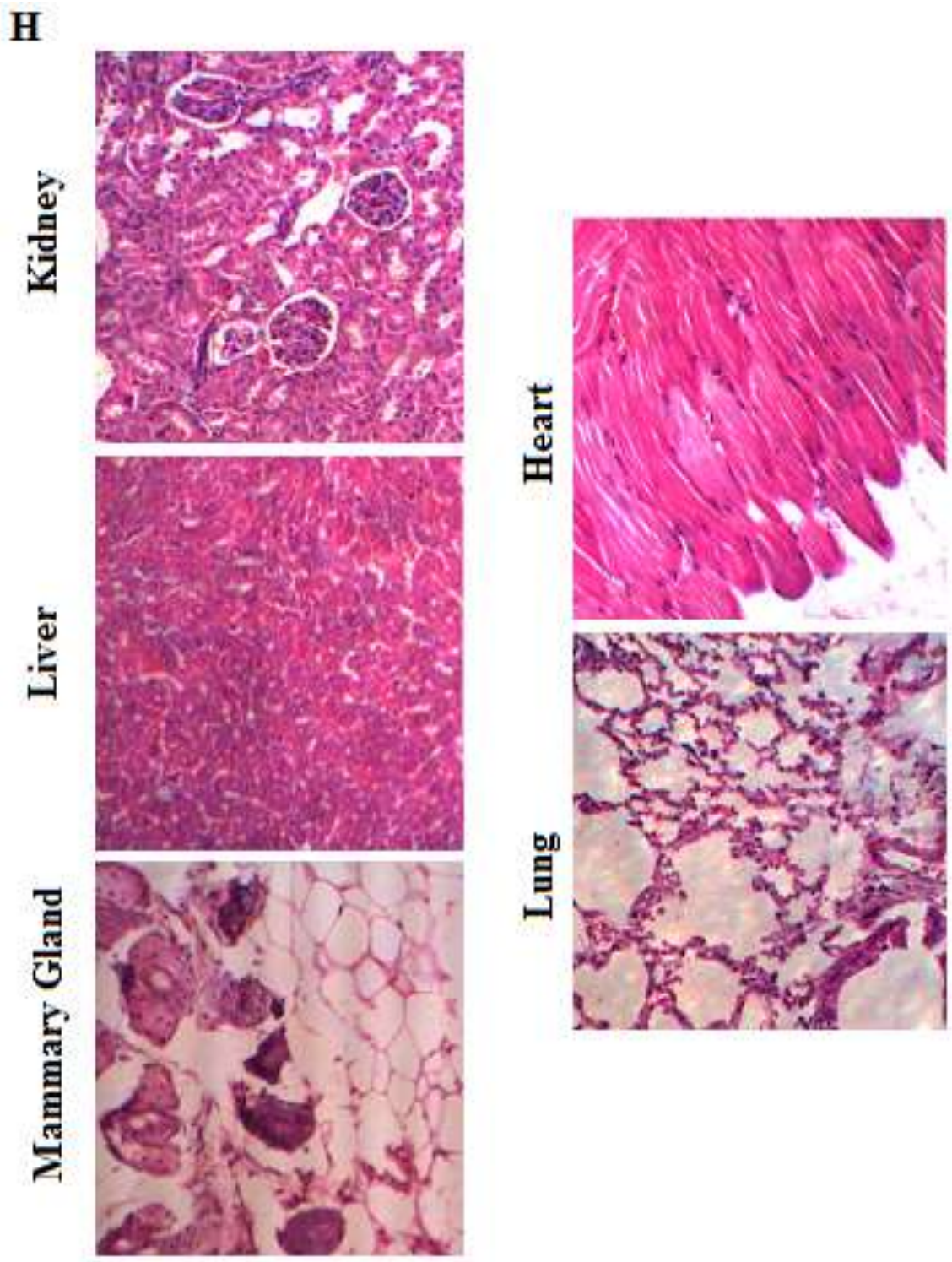

Figure 8.9: Histopathological examination of various organs of normal control (A), model control (B), standard control (C), DOX-MSN (D), DOX-MSN-SS-CH-FA (E), CuO-MSN (F), DOX-CuO-MSN (G), and DOX-CuO-MSN-SS-CH-FA (H).

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