Supplementary Information

Dose Ranging, Expanded Acute Toxicity and Safety Pharmacology studies for Intravenously Administered Functionalized Graphene Nanoparticle Formulations

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Characterization

Table S1. Physicochemical characterization and *in vitro* study results of GNP-Dex formulation.[1-3]
Abbreviations: TEM, transmission electron microscopy; AFM, atomic force microscopy; EELS, electron energy loss spectroscopy; RS, Raman spectroscopy; ICP-MS, inductively coupled plasma mass spectrometry; EPR, electron paramagnetic resonance; SEM, scanning electron microscopy; TGA, thermal gravimetric analysis.

Study/ Reference	Test Article/Dose	Duration	Evaluation Method	Noteworthy Findings
Particle size	GNP-Dex		TEM, AFM	Size ~100 nm, Thickness 3-4 nm
Size distribution	GNP-Dex		TEM	100±20 nm
Dispersibility	GNP-Dex	0.4, 10, 2, 50, 100, 200 mg/mL	Visual assessment	Stable colloidal dispersion up to 100 mg/ml.
Structure/shape,	GNP-Dex		TEM, AFM	Disc shaped particles
Chemical composition	GNP-Dex		EELS, RS, ICP-MS,EPR	
Topology	GNP-Dex		TEM,SEM, AFM	Dextran coils around the graphene nanoparticles
Quantitative composition	GNP-Dex		ICP-MS TGA	GNP-60% by weight. Dextran-40% by weight. Manganese – 0.064 % by weight.
Surface coating and composition	GNP-Dex		TGA	Dextran 10K; 40% by weight
Stability - chemical-thermal stability	GNP-Dex 20, 50, and 100 mg/mL	3 and 24 hours, 30 days @ 25^{o}C and 37^{o}C	$NaBiO_3$ test- UV-Vis spectrophotometer	No color change observed in sodium bismuthate ($NaBiO_3$) test. Negligible < Limit Of Detection (0.01µM) of UV-Vis spectrophotometer.
Dispersion stability	GNP-Dex 0.4, 10, 20,	0, 1, 2, 4, 24 hours	Visual assessment and digital	No apparent precipitation

	50, and 100 mg/mL in Distilled Deionized water + mannitol (55 mg/ml)	after formulation preparation	photograph of the solution	through 4 hours - all concentrations. Apparent precipitation at 100 mg/kg at 24 hour, but easily redispersed.
Osmolality	0.4-100 mg/mL in DDI water	*In vitro*	Osmometer	Hypo-osmolar in DDI water (2-186 mOsm). With addition of Mannitol in GNP-Dex solution roughly iso-osmolar range to blood (190-320 mOsm/kg).
Viscosity	0.4-100 mg/mL	*In Vitro*	Viscometer	1.01-3.81 cP @ 37^{o}C, 50 rpm and 0.86-2.09 cP @ 37^{o}C, 100 rpm; values similar to blood viscosity (3-4 cp) and generally other approved MRI agents.
Hydrophilicity/partition co-efficient	20 mg/mL	*In Vitro*	Flask shaking method and analysis of nanoparticle concentration by spectro-photometry	Very hydrophilic with low partition coefficient; log K_{ow} = -0.18.

Table S2. *In vitro* and *in vivo* studies of GNP-Dex formulation.[2, 3]

Abbreviations: ELISA,enzyme-linked immunosorbent assay; OPT, o-phthalaldehyde**;** Rxn, reaction; PEI, Polyethylene imine; RBC, red blood cells; ACh, acetylcholine; ADO, adenosine; PE, phenylephrine; PF4, platelet factor 4; Sc5b9, Soluble terminal Complement Complex; Bb, factor B.

Study/ Reference	**Species/ Test System**	**Test Article/Dose**	**Duration**	**Route**	**Evaluation**	**Noteworthy Findings**
Protein Binding	Human albumin	0.1,1 and 10 mg/mL	37^{o}C for 24 hours	*In vitro*	Equilibrium dialysis and UV-Vis spectrophotometer	GNP-Dex did not show significant binding to protein.
Histamine Release	Whole blood, human	0.1, 1, and 10 mg/mL – GNP-Dex	60 min at 37^{o}C	*In vitro*	Histamine levels (test kit) – ELISA	Negligible ↑ in level of histamine release up to 0.1 mg/mL when compared with controls.
	Mast cells, rat	0, 1, 7, and 10 mg/mL GNP-Dex	5 min pre-treatment		Histamine-OPT Rxn – Fluorescence intensity	Negligible ↑ in level of histamine release up to 10 mg/mL when compared with controls.
Platelet Activation	Whole blood, human – 2 donors	0, 1, 7, and 10 mg/mL GNP-Dex	45 min pre-treatment	*In vitro*	PF4 release - ELISA	Negligible ↑ in level of PF4 up to 10 mg/mL when compared with controls.

Study/ Reference	**Species/ Test System**	**Test Article/Dose**	**Duration**	**Route**	**Evaluation**	**Noteworthy Findings**
Complement Activation	Whole blood, human-2 donors	0, 1, 7, and 10 mg/mL GNP-Dex	45 min pre-treatment	*In vitro*	Sc5b9 or Bb levels (test kit) – ELISA	No significant ↑ in Sc5b9 at 1 and 7 mg/mL GNP-Dex compared with controls. Significant ↑ (≈20% compared with controls) at 10 mg/mL. Similar ↑ (≈18%) in Bb at 4 mg/mL dextran but not at 0.4 or 2.8 mg/mL when compared with controls. ↑ in complement activation at high concentration likely a function of dextran.

Study/ Reference	**Species/ Test System**	**Test Article/Dose**	**Duration**	**Route**	**Evaluation**	**Noteworthy Findings**
RBC Aggregation	Whole blood, human-1 donor	0, 1, 7, and 10 mg/mL GNP-Dex Positive control - PEI	45 min pre-treatment	*In vitro*	Visualization (630X) of blood smears	No aggregation or morphological change in RBCs up to 10 mg/mL. PEI caused aggregation.
Vasoactivity	Hamster; n = 8M/GNP-Dex; 3M/dextran)	Vasoactivity GNP-Dex – 0, 0.1, 0.5, 2.6, 10, and 50 mg/mL Dextran – 3.5, 35 mg/mL		IV	Cheek pouch model – vasoactivity; ACh, ADO, PE response modification - arcade and terminal arterioles junction - Δ in vessel diameter in response to vasoactive compounds	Concentration dependent dilation with GNP-Dex; significant ↑ ≥ 2.4 mg/mL; EC50 = 2.4-2.6 mg/mL with maximum dilation ranging from 60% (arcade arterioles) to

Study/ Reference	Species/ Test System	Test Article/Dose	Duration	Route	Evaluation	Noteworthy Findings
		Vasoresponse change – GNP-Dex - 50 mg/mL; pre and 15-min post vasoactive agent exposure 4-10M ACh, ADO, PE				76% (terminal arterioles); no effect with dextran only. No apparent effect on vasoresponse to ACh, ADO, PE at doses of 50 mg/mL. Conclusion: No endothelial dysfunction associated with exposure to GNP-Dex based on an absence of alteration in response to vasoactive agents.

Table S3. Rationale for choosing the medium and high GNP-Dex doses. The potential clinical therapeutic doses of graphene-based formulations for potential imaging or therapeutic applications still need to be determined. Therefore, published values of other clinical imaging and therapeutic agents; shown in the table below, were used as representative examples. We assumed that GNP-Dex could also be eventually administered, as a therapeutic or imaging agent, at similar doses. Based on this assumption and published regulatory guidelines, we chose medium to high doses 10-100 times the doses of these other clinical approved pharmaceuticals. The upper limit (500 mg/kg) was limited by the maximum permissible dose (MPD) that could be achieved using the highest GNP-Dex stock concentration of 100 mg/ml.

Clinically approve therapeutic and diagnostic agents	**Recommended clinical dose**	**Equivalent mg/kg dose**
Myocet - Liposomal doxorubicin	60 -75 mg/m^2 [4][4]	1.632-2.04 mg/kg*
Abraxane – Albumin bound Paclitaxel nanoparticles	100-250 mg/m^2 [5][5]	2.72 -6.8 mg/kg*
Doxorubicin - chemotherapeutic	60 mg/m^2 [6][6]	1.632 mg/kg*
Ablavar – Blood pool magnetic resonance imaging contrast agent	0.03 mmol/kg [7][7]	29 mg/kg

*Note: The conversion to mg/kg doses were performed based on the body weight and body surface area information provided at following webpage.

http://www.vspn.org/Library/Misc/VSPN_M02372.htm

Extrapolation of % of GNP-Dex

As reported in our previous study [2], the % of manganese calculated in the GNP-Dex samples was average of 6 different samples of GNP-Dex. The ICP MS system used in this study allows detection even at concentrations down to1 ppb.

For the biodistribution studies, with the tested three doses 50, 250 and 500 mg/kg, a total of 12.5, 62.5 and 125 mg of GNP-Dex injected in a 250 g rat. Considering 0.064 wt% of manganese in the sample, the amount of manganese injected in rats will be 8, 40 and 80 µg for 50, 250 and 500 mg/kg respectively. Thus, 8, 40 and 80 µg of manganese was considered as 100% injected dose in each animal injected with 50, 250 and 500 mg/kg respectively.

Since tissue samples were pooled from 6 animals for chemical digestion, the measured manganese i.e. [Mn^{2+} (experimental group)] = [concentration obtained from ICP]/6.

Similarly, the measured Mn^{2+} for tissue sample pooled from 6 sham animals i.e. [(Mn^{2+} (sham control group)] = [concentration obtained from ICP]/6.

The % injected dose values in various tissues were calculated using the formula

[{[Mn^{2+} (experimental group)] - [(Mn^{2+} (sham control group)}]/ [Mn^{2+} (injected doses)]]* 100.

Necropsy

Necropsies were performed on five rats postmortem. The procedure was performed by Antech Diagnostics (New Hyde Park, NY). The outcomes and diagnosis from all the necropsies were similar. The pdf copy of one necropsy report titled “necropsy” is uploaded as a separate file.

Figure Legends

Figure S1. Representative TEM image of GNP-Dex **a)** low magnification and **b)** higher magnification.

Figure S2. Digital image of GNP-Dex formulations used in toxicity study for animal injections at 100 to 0.4 mg/ml in distilled deionized water.

Figure S3. Representative histologic sections of a day 1 animal at 500 mg/kg of GNP-Dex dose at 400x magnification a) Cerebellum: without any diagnostic abnormality. b) Heart: without diagnostic abnormalities. c) Liver: hepatic parenchyma with amorphous debris within sinusoids and central vein (circle). A solitary aggregate of neutrophils was noted (arrow), however, there was no evidence of acute or chronic inflammation. d) Lung: pulmonary parenchyma with vascular congestion and intracapillary aggregates of brown pigments (circle). e) Kidney: focal aggregates of brown/black pigments in veins (circle) and glomeruli with mild congestion (arrow).

Figure S4. Representative tissue histologic sections of a day 30 animal at 500 mg/kg of GNP-Dex dose at 400x magnification a) Cerebellum: without any diagnostic abnormality. b) Heart: without diagnostic abnormalities. c) Lliver: with patchy centrilobular congestion (arrow), but no other diagnostic abnormalities. d) Lung: focal extravasation of red blood cells into alveolar spaces (arrow). e) Kidney: renal medulla congestion (arrows) and aggregates of granular pigments within vascular spaces (circle).

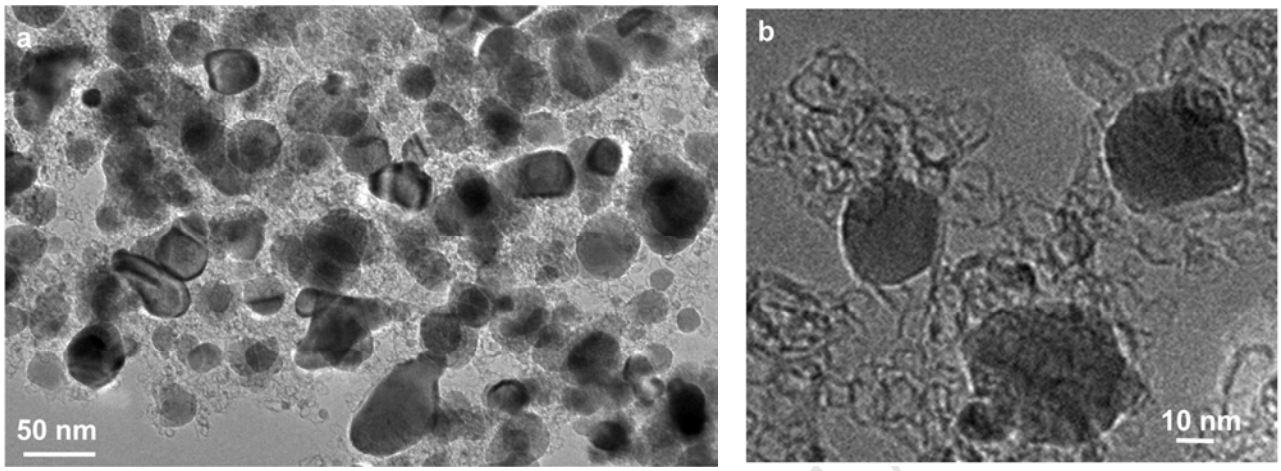
Figure S5. Representative tissue histologic sections of a day 30 animal at 250 mg/kg of GNP-Dex dose at 400x magnification a) Cerebral cortex without any diagnostic abnormality. b) Myocardium without diagnostic abnormalities. c) Liver: hepatic parenchyma with amorphous debris in central vein suggestive of presence of GNP-Dex (arrow), no evidence of inflammation. d) Lung: pulmonary parenchyma without any diagnostic abnormalities. e) Kidney: renal cortex with vascular congestion (arrows).

Figure S6. Hematological parameters obtained from blood pressure monitor and echocardiography 10 min and 2 hours post injection of GNP-Dex. **a**) Left ventricular volume, diastolic. **b**) Left ventricular volume, systolic. **c**) Atrioventricular peak velocity. **d**) % fractional shortening. **e**) Intravascular septum thickness, diastolic. **f**) Intravascular septum thickness, systolic. **g**) Left ventricular internal dimension, diastolic. **h**) Left ventricular internal dimension, systolic. **i**) Left ventricular posterior wall thickness, diastolic. **j**) Left ventricular posterior wall thickness, systolic.

Figure S7. Blood chemistry results for rats injected with GNP-Dex, dextran and mannitol 1 day after injection (n=3). **a**) Blood lipid levels of triglycerides (TG), cholesterol (CHO) and blood glucose (GLU) b) Blood ion concentrations of Na, K, Cl and CO_2 **c**) Blood protein levels of total protein (TP) and albumin. All parameters were within the normal range.

Figures

Figure S1



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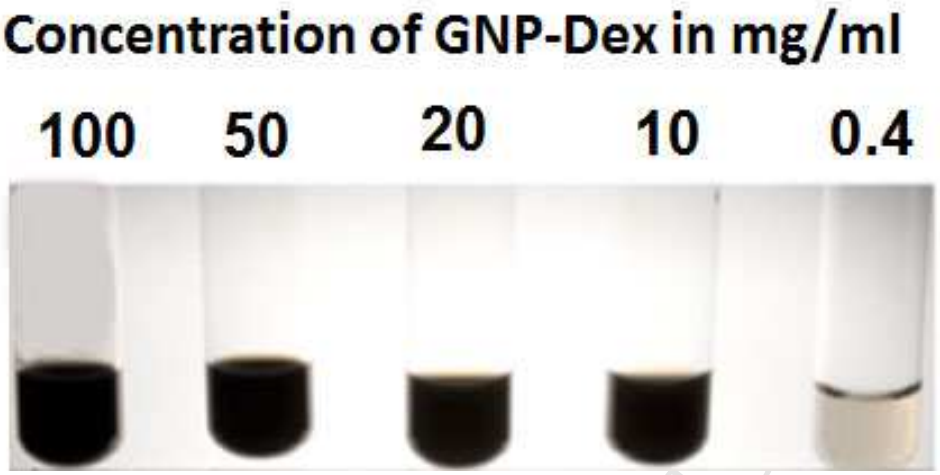
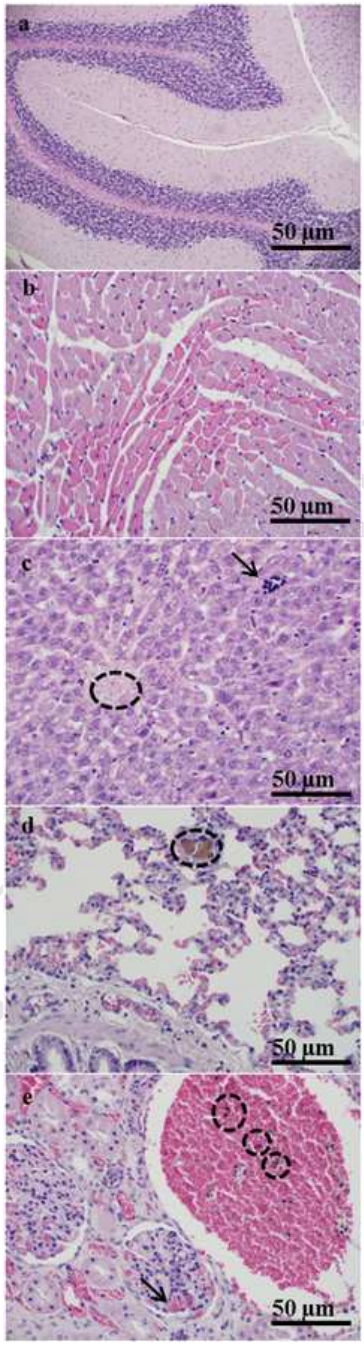


Histopathology

Figure S3



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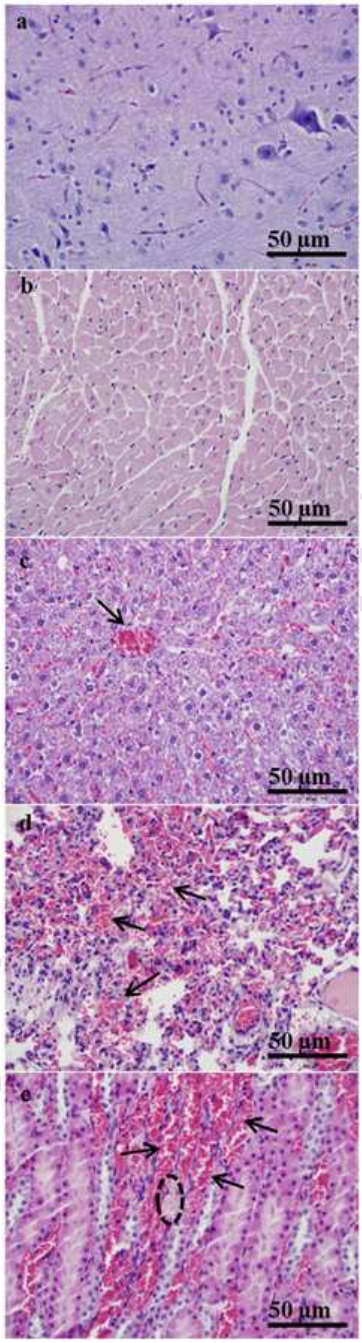

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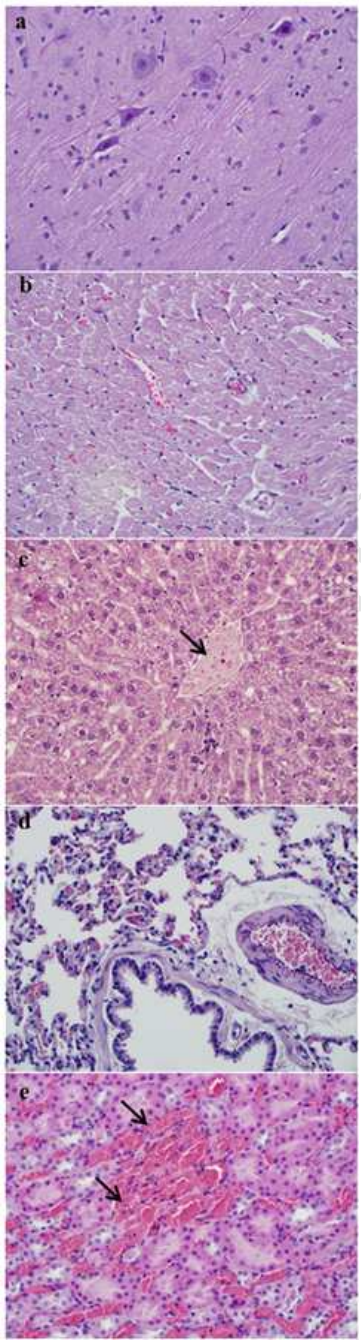


Hematological parameters

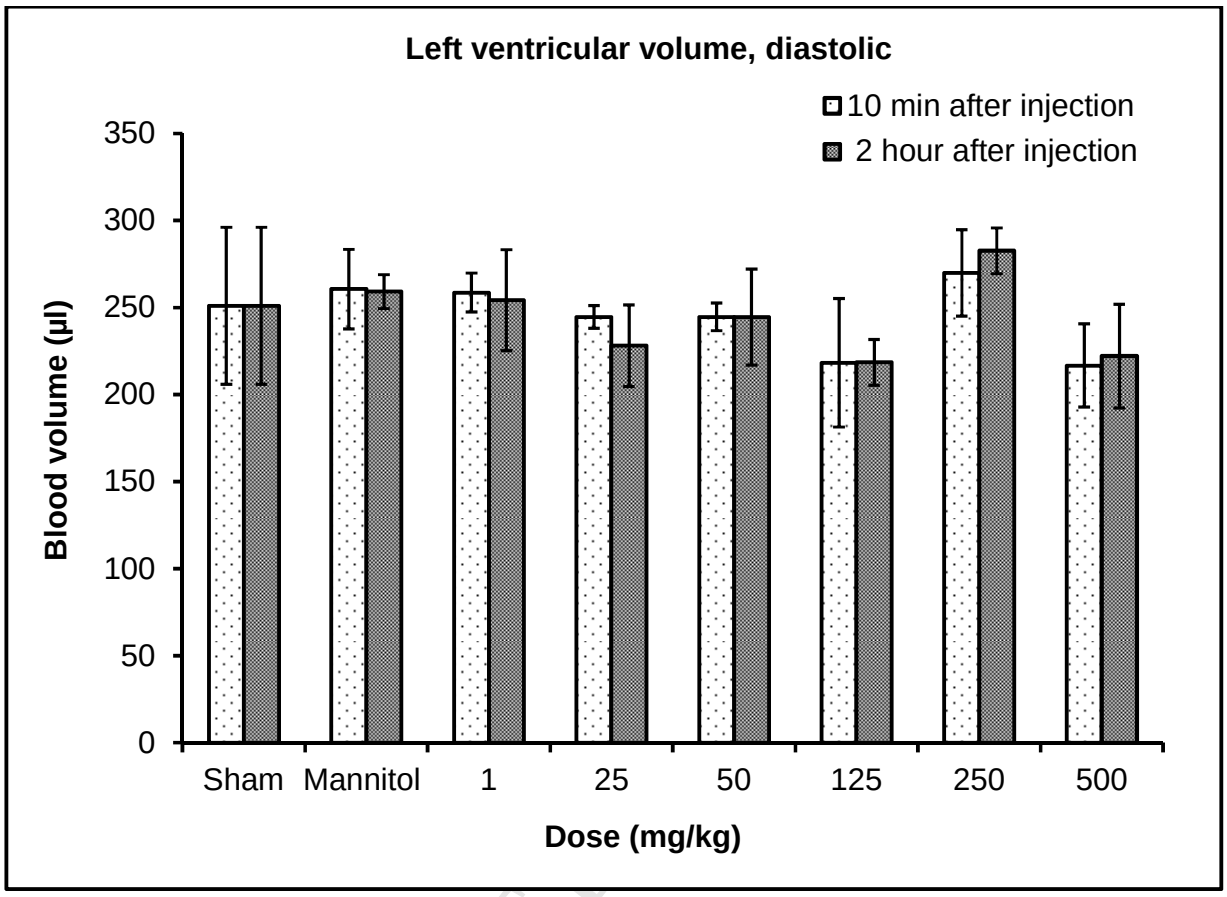
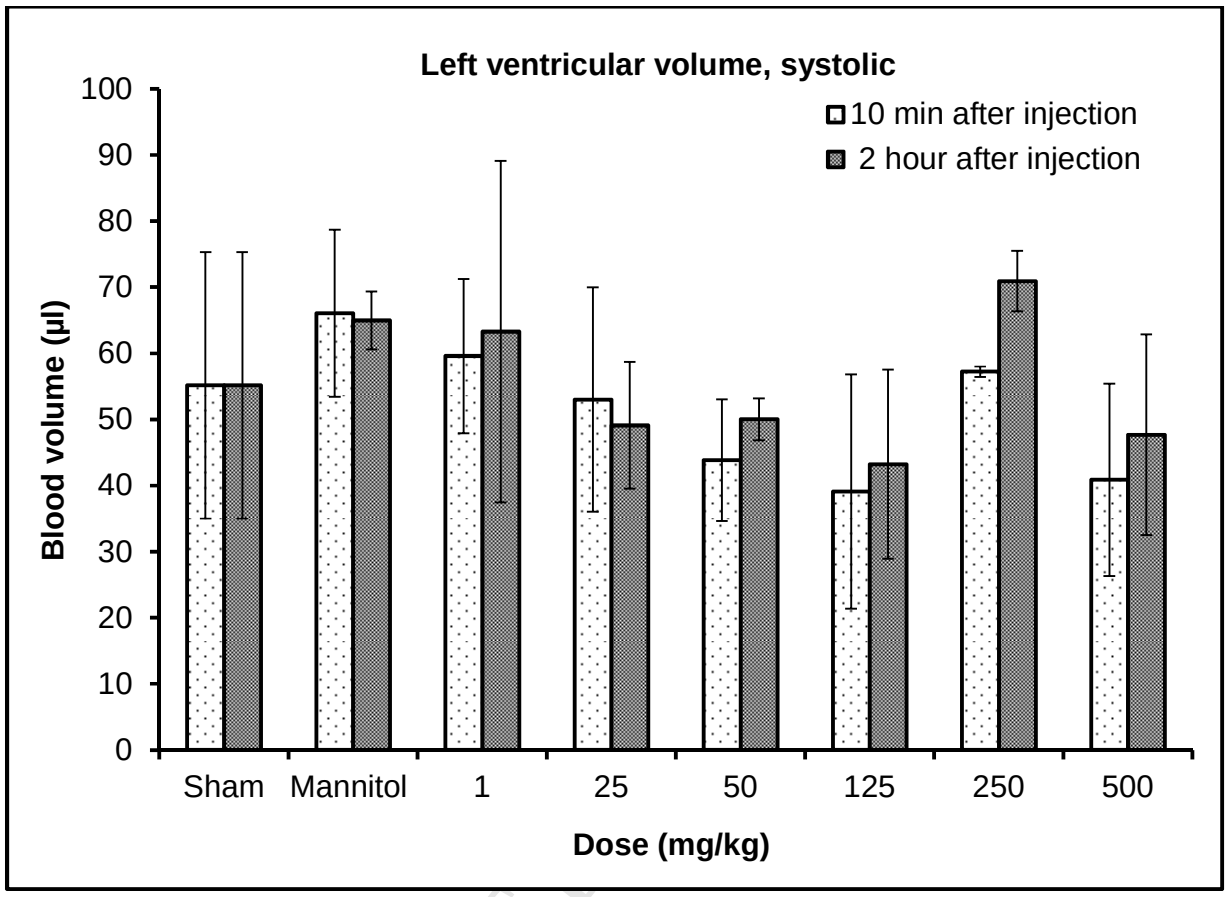


Figure S6 b



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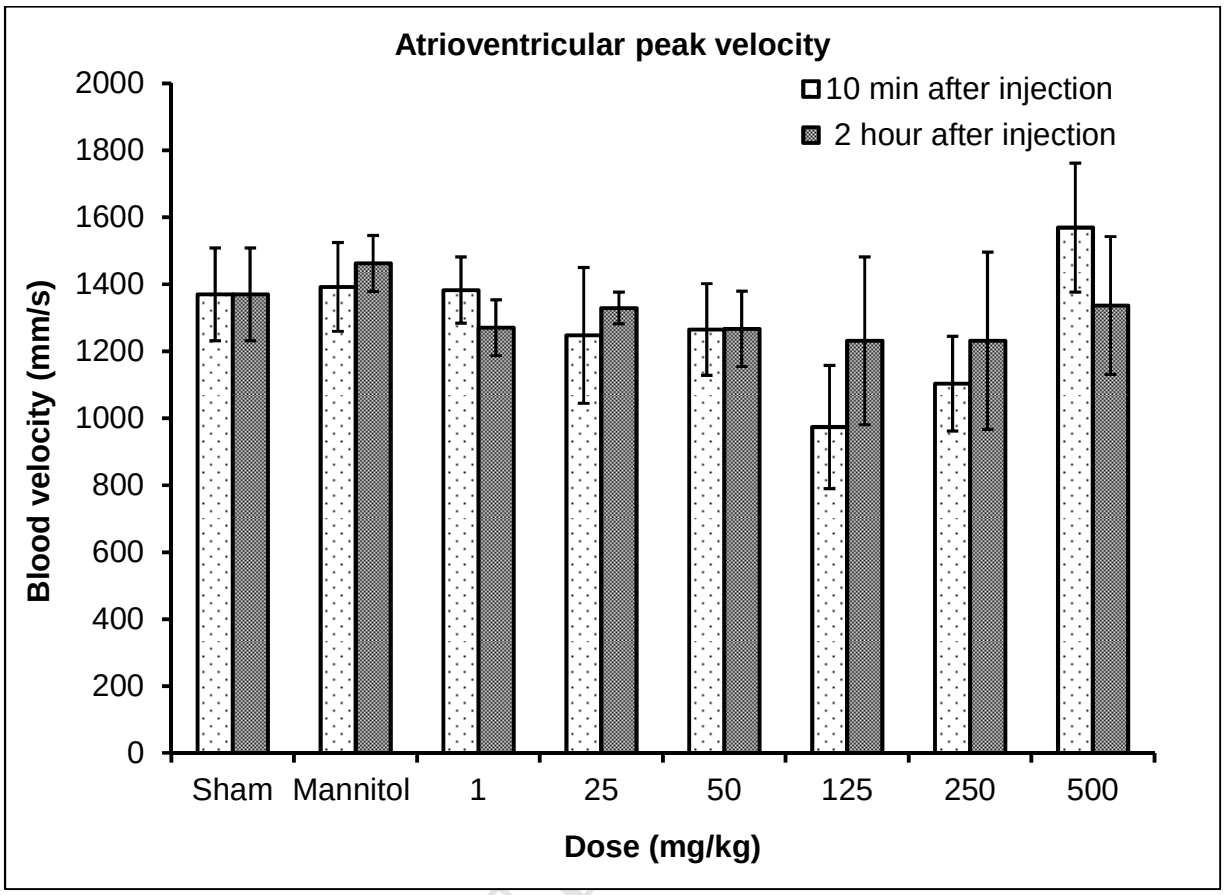

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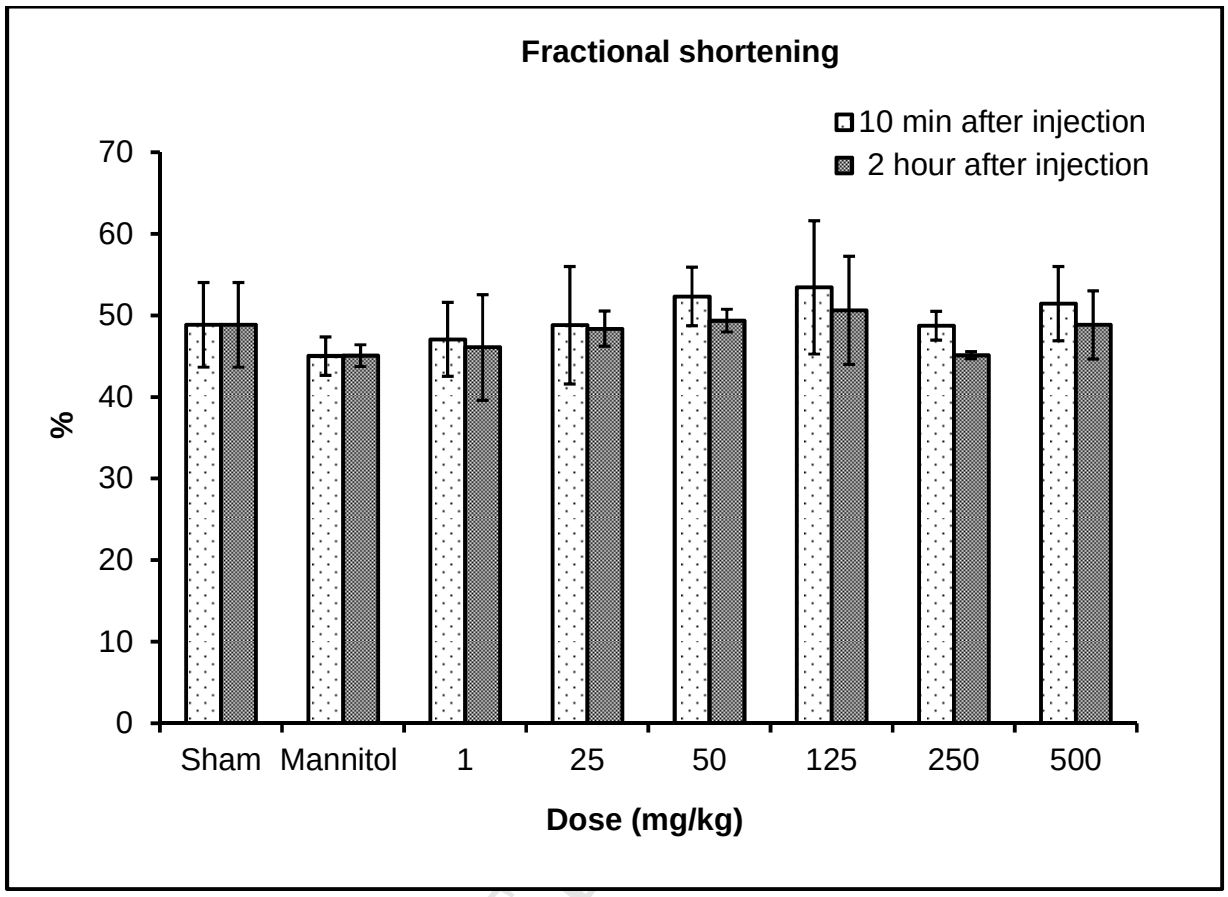

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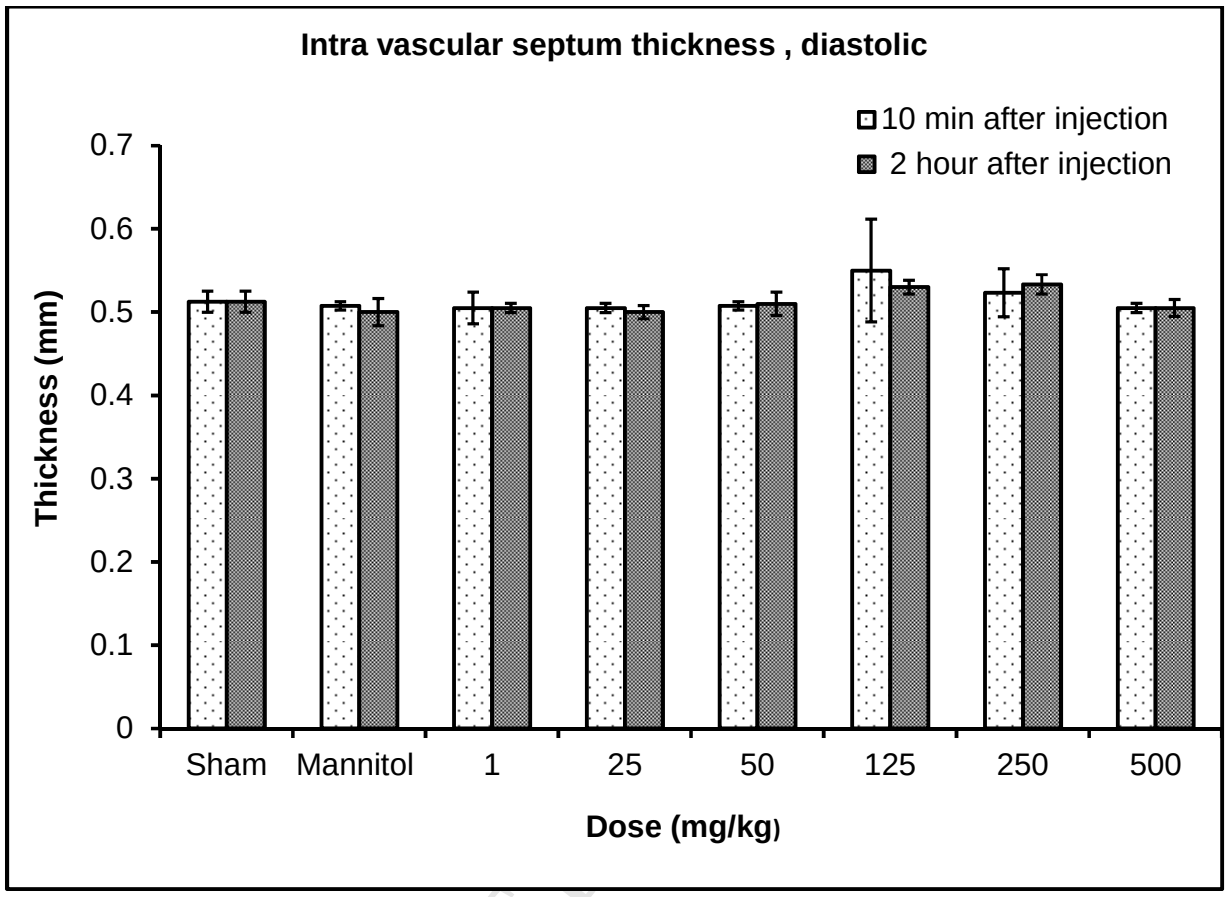

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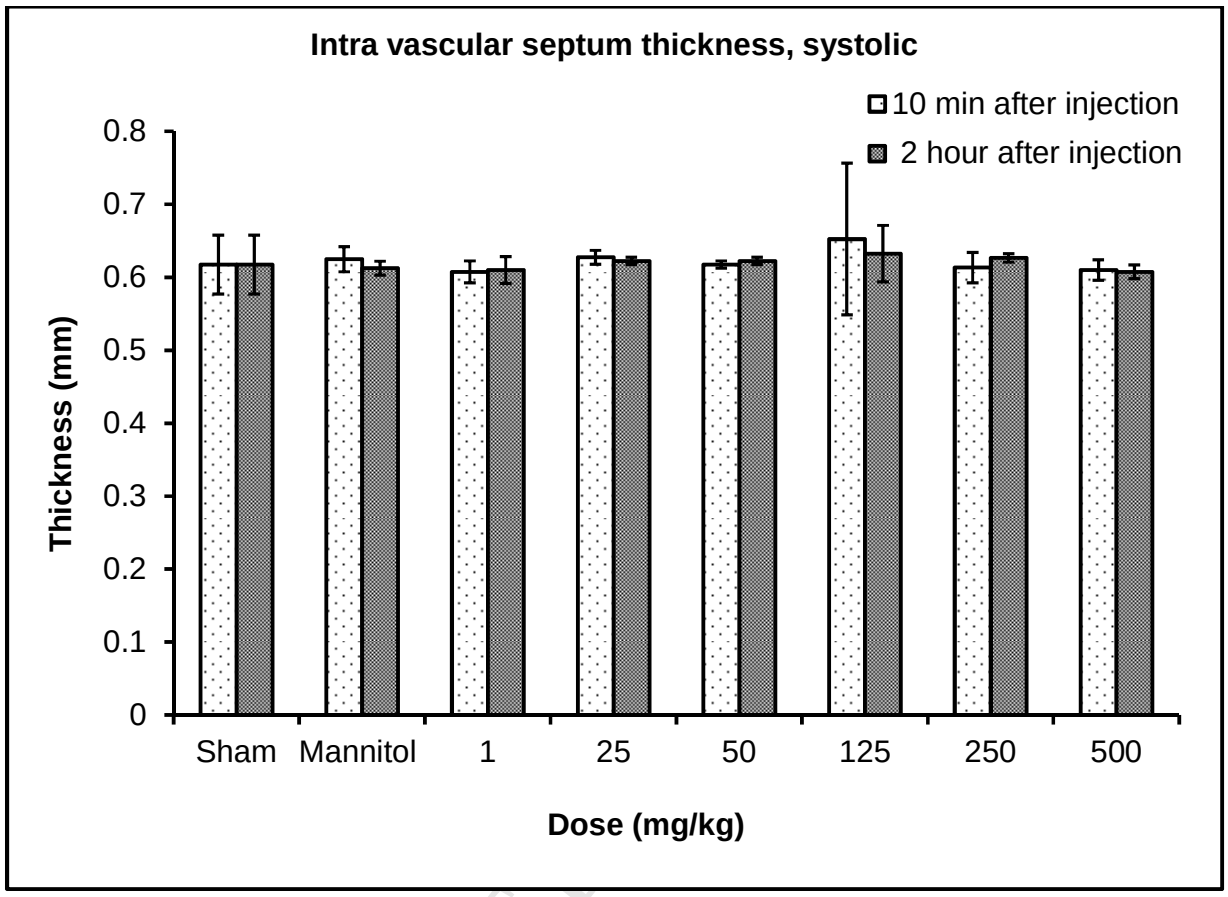

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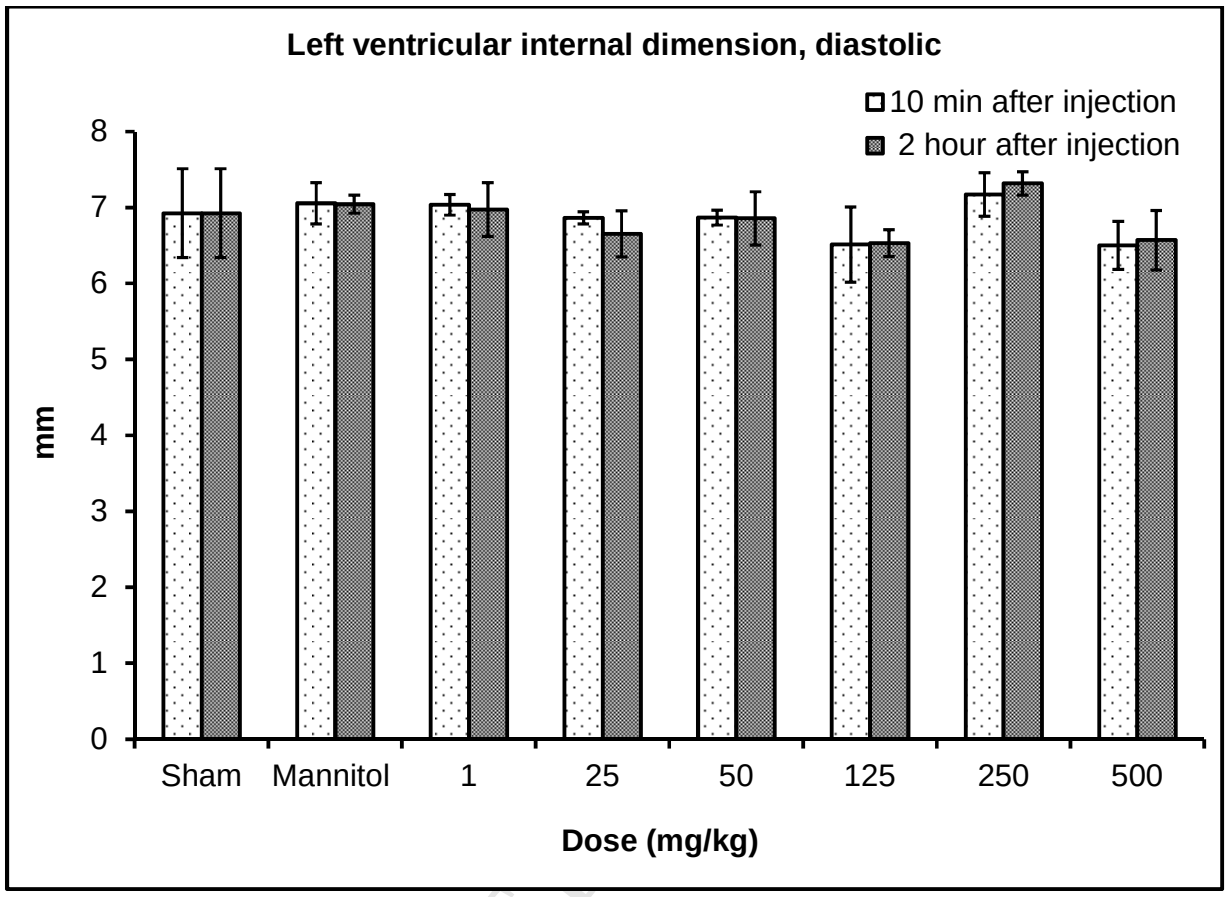


Figure S6 h

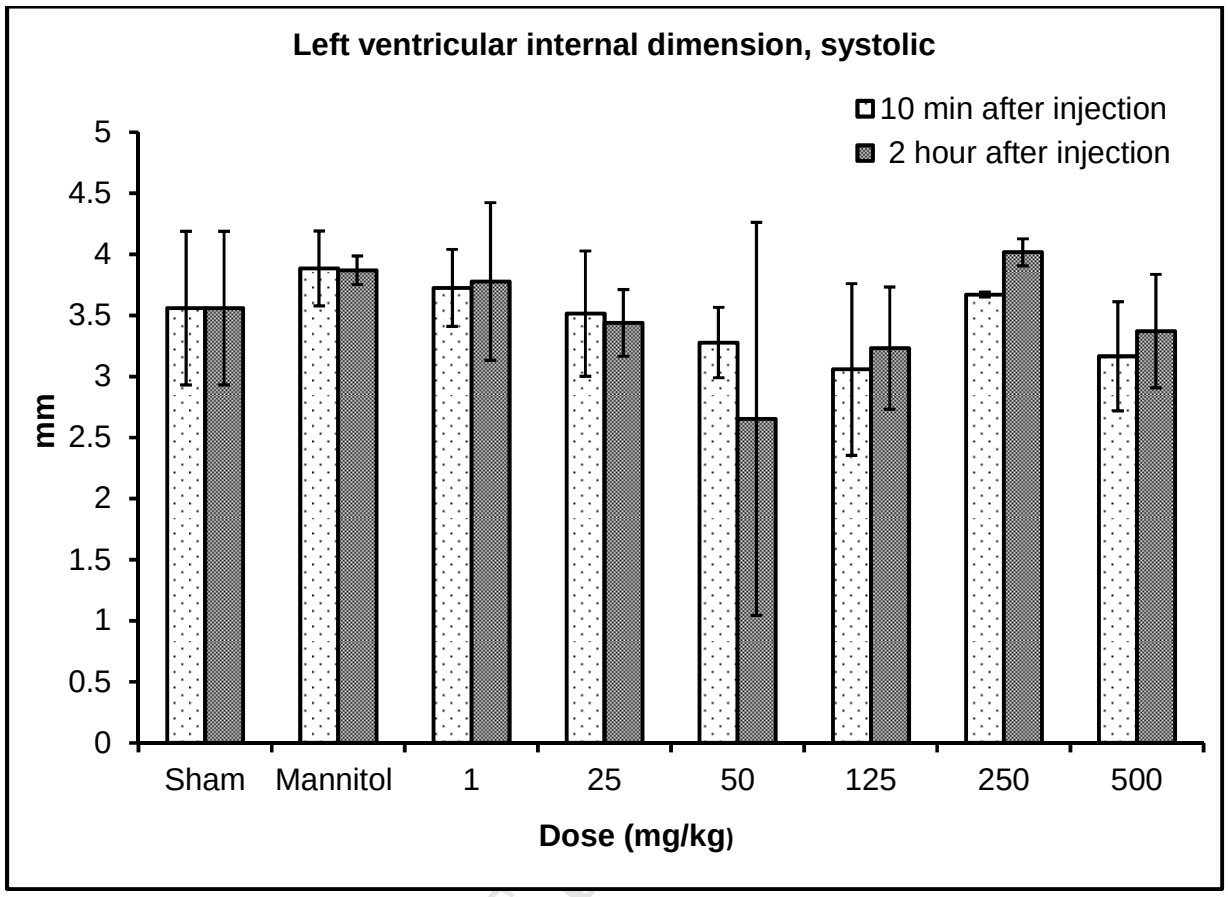
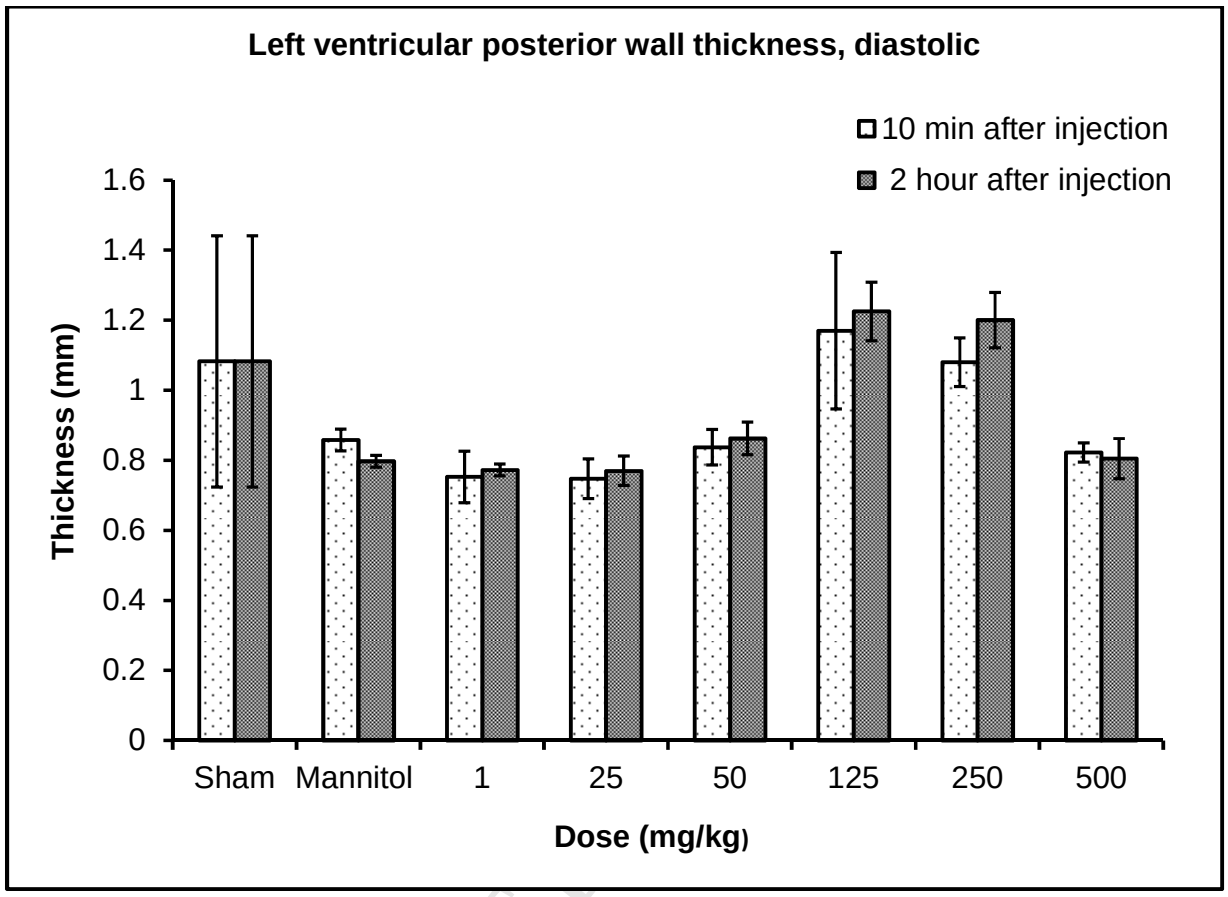


Figure S6 i



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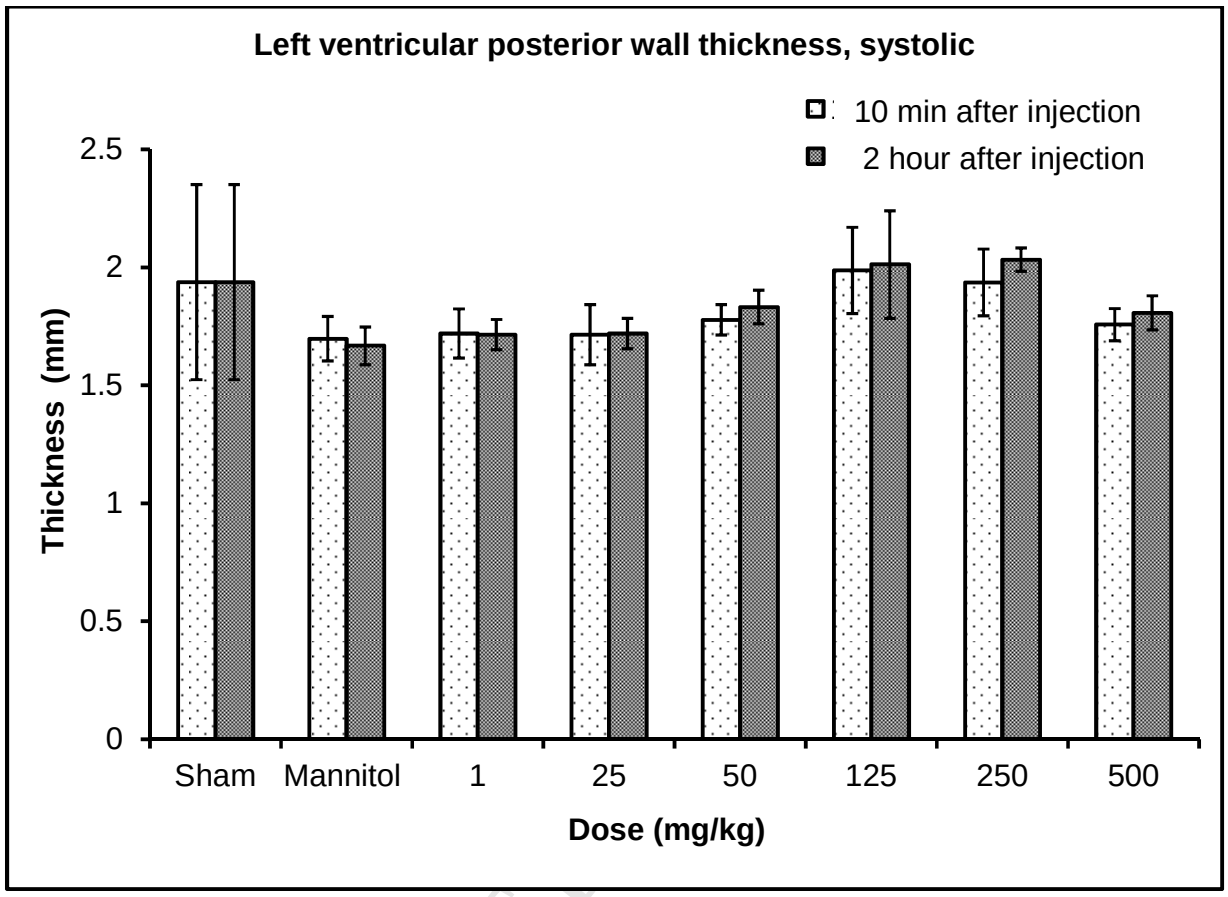
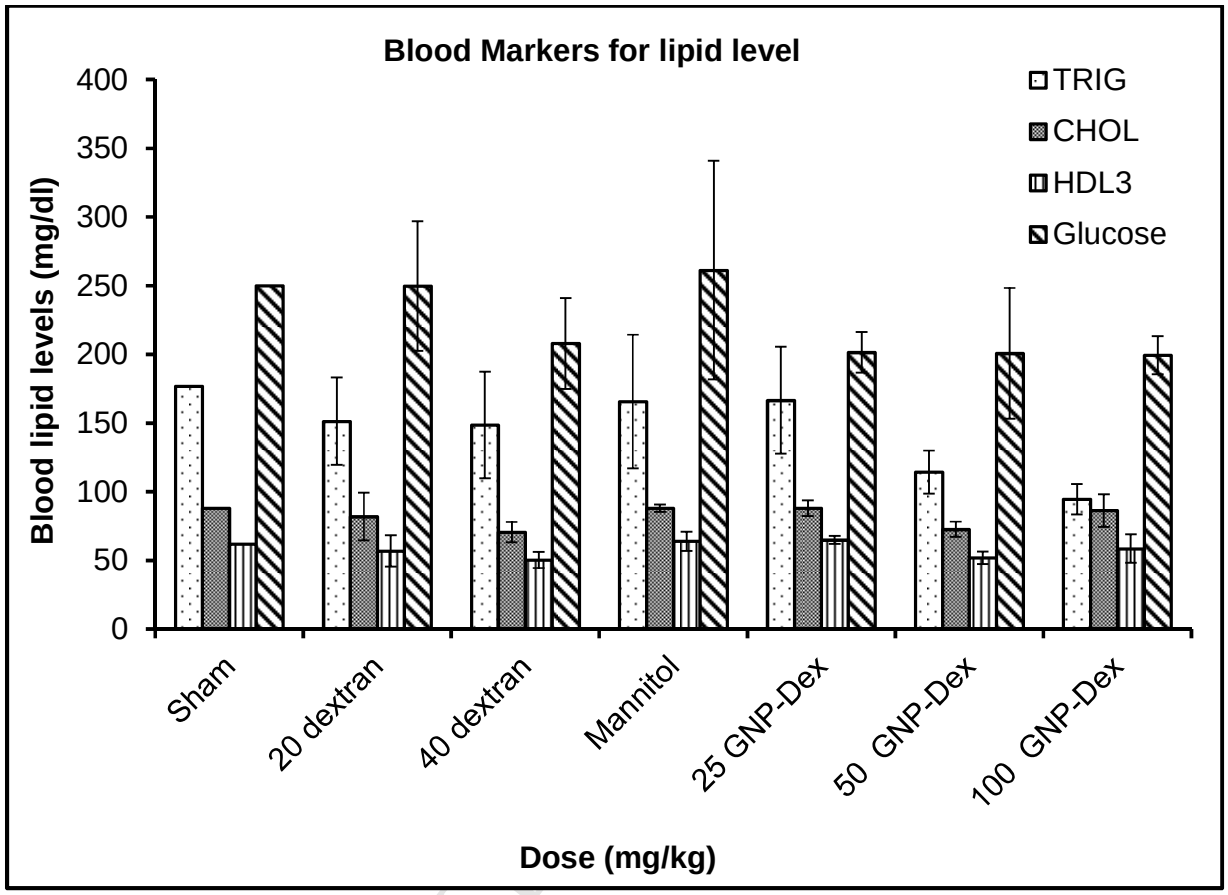


Blood chemistry

Figure S7 a



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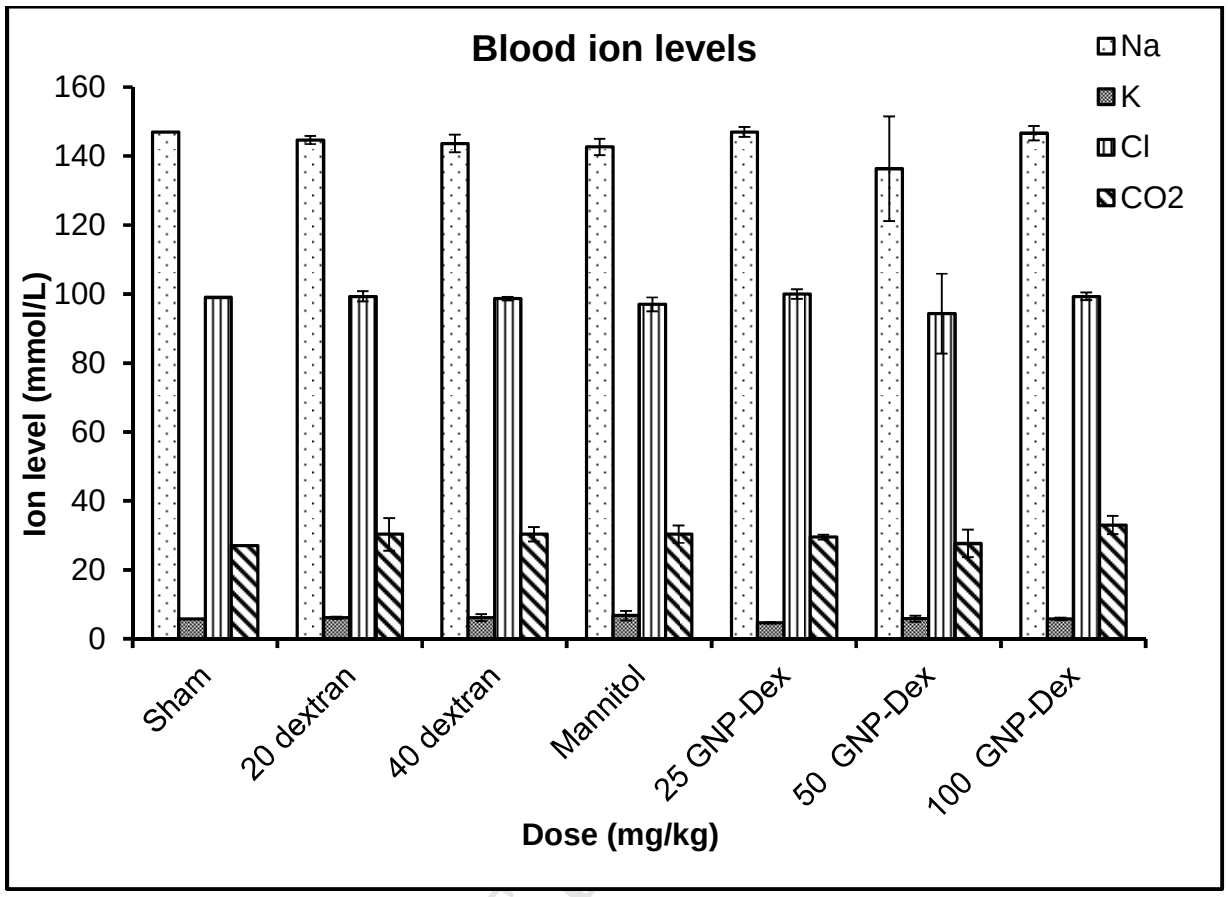

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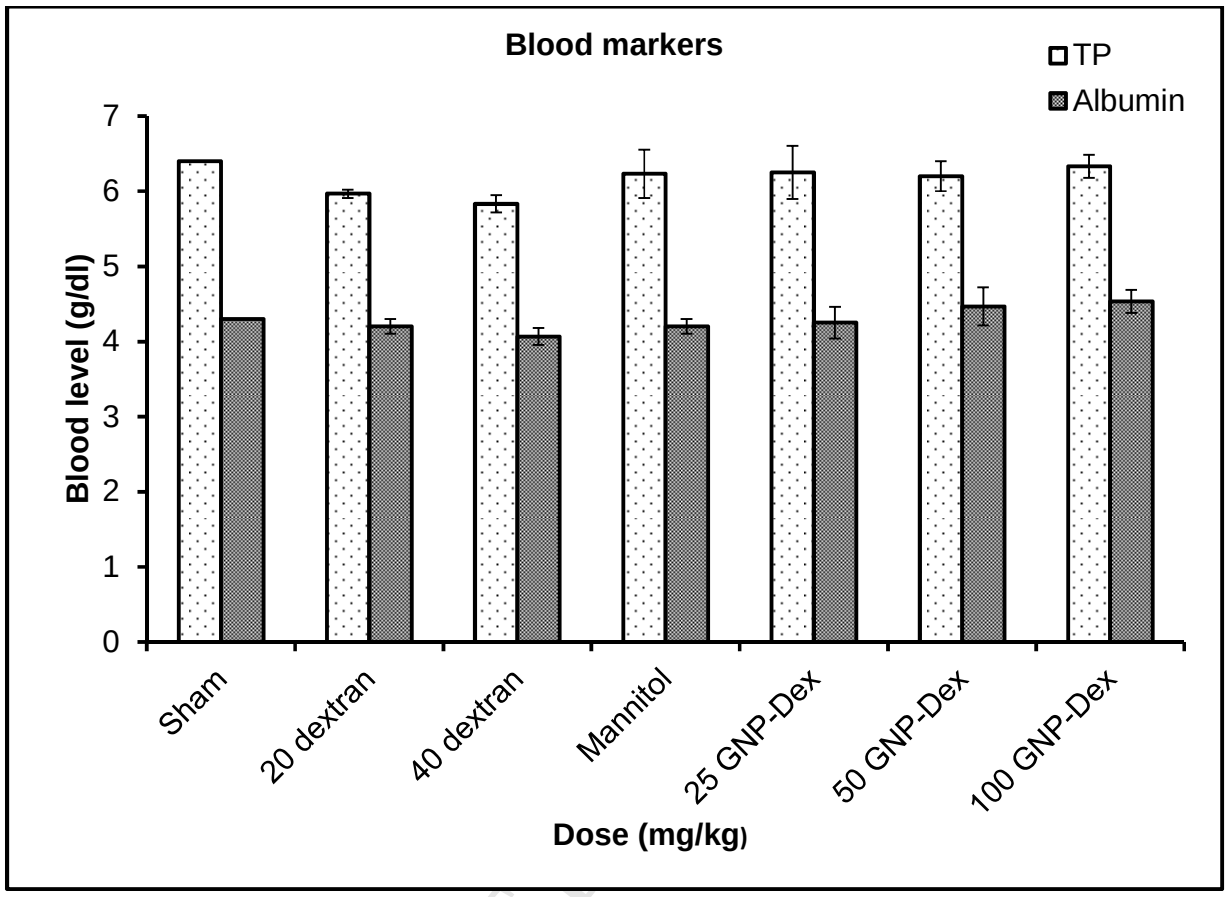

References

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