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Biocompatibility and Genotoxicity Studies of Polyallylamine Hydrochloride Nanocapsules in Rats

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Abstract

Polymer nanocapsules have attracted a great deal of interest for drug delivery and bioimaging applications owing to their functional versatility. The present study focusses on synthesis, characterisation, biocompatibility and genotoxicity studies of polyallylamine hydrochloride (PAH) nanocapsules for drug delivery studies. *In vitro* studies included are haemobiocompactibility studies, cytotoxicity and comet assay in peripheral blood mononuclear cells (PBMCs). Post intravenous administration of PAH nanocapsules, alteration in haematological parameters, inflammatory marker status, toxicity markers in serum and major organs, RT-PCR, Western blotting and histopathological studies of major tissues of rat were evaluated for 30 days. Results of these *in vitro* studies indicated biocompatible nature of the PAH nanocapsules at the tested concentration (1.5×10^5- 6.0×10^5capsules/ml). *In vivo* toxicity markers activity, inflammatory marker status like cyclooxygenase (COX), lipooxygenase (LOX), nitric oxide synthase (NOS) and prostaglandin E2 (PGE2) activity, haematological parameters alteration and RT-PCR analysis of important genes like interleukin1beta (IL-1β), monocyte chemotactic protein-1(MCP-1), transforming growth factor beta 1 (TGF- β1), kidney injury molecule-1(Kim-1), heat shock protein gene (Hsp70-1) and tumor necrosis factor alpha (TNF-α) showed least changes. Western blotting studies on immunoregulatory proteins like cytokines (IL-8), chemokines (MIP-2) and cell adhesion molecules (VCAM-1and ICAM 1) showed least level of toxicity with PAH nanocapsules interaction. Histopathological studies of important tissues showed almost normal architecture after treatment with PAH nanocapsules throughout the experimental period. The above results confirm the biocompatibility and non toxicity of PAH nanocapsules, thus suggesting their potential for *in vivo* drug delivery and bioimaging applications.

Key words; Biocompatibility; Immunoregulatory; PAH Nanocapsules; Genotoxicity

Introduction

Nanocapsules hold significant biomedical interest owing to their nanometer size-scale and tunable properties like controlled release and targeting of drugs towards specific sites.[1] Owing to their versatility they can be endowed with multiple functionalities and thus can be used for a variety of biomedical applications, nanocapsules possess greater capability to be taken over an extensive range of applications with extremely high efficient reproducibility.[2] Polymeric nanocapsules have been fabricated by sacrificial template based approaches either through step-wise adsorption of polymers using electrostatics/H-bonding/covalent chemistry etc as driving force or polymer infiltration into mesoporous shell of the core and followed by the dissolution of the core template. [3, 4] The latter approach of nanocapsule synthesis is particularly attractive from the standpoint of single step assembly of macromolecules as opposed to multiple steps involved in layer-by-layer assembly. Thereby this route is more suitable for large scale nanocapsule synthesis along with simpler control over the physical properties of the capsules.

Recently, Wang *et al* reported polymer infiltration mediated synthesis of nanocapsules based on both polypeptides like poly(L-glutamic acid) and synthetic polyelectrolytes like polyallylamine hydrochloride (PAH).[5] Doxorubicin loaded poly(L-glutamic acid) nanocapsules showed promising delivery of anticancer drug to tumor cells *in vitro*, indicating their potential for drug delivery applications. However, PAH nanocapsules were not investigated for their *in vitro* and *in vivo* biocompatibility profile. Moreover, PAH based nanomaterials have been extensively synthesized for prospective drug delivery and bioimaging applications but studies on their detailed biocompatibility profile are still lacking. To this end, we synthesized PAH nanocapsules and subjected them to comprehensive biocompatibility and genotoxicity testing within an *in vitro* and *in vivo* environment. These PAH nanocapsules can serve as efficient carriers for drug delivery and bioimaging agents for diseases like atherosclerosis.

Atherosclerosis is a multifunctional disease characterized by oxidative, inflammatory, immunologic, and necrotic processes.[6] Current strategies to treat atherosclerosis include treatment by statins, which decrease cholesterol synthesis resulting in increased uptake of dietary cholesterol carried by Low-density lipoproteins (LDLs) [7] surgical intervention by angioplasty procedures that may include stents to physically expand narrowed arteries. Macrophages are primary cells which present in the site of atherosclerotic plaque and the major factor for the development of atherosclerosis. Its activation results in the excretion of

proinflammatory and cytotoxicity substances, including peroxynitrite, an early inducer of atherosclerosis through the endoplasmic reticulum (ER) stress pathway.[8, 9] Macrophage apoptosis suppresses atherosclerotic lesion size. [10] Detection of macrophage apoptosis seems to be a rare event in early atherosclerotic lesions, but is observed in and around the necrotic core of advanced lesions. [11] Early therapy is difficult as finding macrophages in the initial stage of atherosclerosis is complicated. Thus, tracking of the initial atherosclerotic lesions by targeted fluorescent nanocapsules combined with drug delivery is a promising application for prospective nanocapsule based therapies.

Increased use of nanocapsules for diverse biomedical applications have raised concerns about their safety in human health.[12,13] Detailed investigation of their haemocompatibility, cell cytotoxicity, immunocompatibility and *in vivo* toxicity is imperative to address these issues and investigate the true *in vivo* potential of these nanocapsules. Recent studies show that multiple assays can be employed, depending on the type of nanomaterials for assessing their cytotoxicity.[14, 15] Moreover, there are very few studies dealing with investigation of polymer capsules for *in vivo* behaviour. For instance, Bulcao *et al* very recently reported the importance of *in vivo* testing of polymeric nanocapsules after intradermal administration.[13]

Hence, aim of the proposed study was to fabricate polyallylamine hydrochloride (PAH) nanocapsules and to know its biocompatibility and genotoxicity in rats for knowing its suitability as a carrier for atherosclerotic drug delivery and imaging applications.

Materials and Methods

All reagents used for the study were of analytical grade. Tetraethoxysilane (TES 28 SQ), n-Octadecyltrimethoxysilane (91.6%), aqueous ammonia (reagent grade, 32 wt.-%), Glutaraldehyde, Poly-allylamine hydrochloride (PAH) Mw 70 kD and Histopaque 1077 purchased from sigma-Aldrich co, St. Louis, USA. All chemicals and reagents used for cytotoxicity analysis experiments were purchased from Sigma, Aldrich, USA. Rest of the chemicals and solvents used were purchased from SRL and spectrochem, India.

Synthesis and characterisation of PAH nanocapsules

The synthesis of PAH nanocapsules was done as per a reported procedure, which is described in brief.[17] Solid core mesoporous shell silica template (10 mg) was taken and polymer was infiltrated into the mesoporous shell by incubating the template particles with PAH solution (5 mg/ml in 0.2 M NaCl; pH 8.5) overnight with gentle mixing. Post infiltration, the unbound polymer was washed off thrice by water. The polymer was then cross-linked by incubating with glutaraldehyde for 20 min and then washed with water. The template was etched out

using 2 M HF: 8 M NH4F (pH 5) (*Caution: HF is very toxic, so should be handled with all safety precautions*) to get nanocapsules. Characterisation of PAH nanocapsules was done by scanning electron microscopy (SEM).

***In vitro* Studies**

Blood compatibility studies

Blood was drawn from healthy Sprague dawely rats and collected in tubes containing 3.8% sodium citrate at ratio 9:1 (blood : anticoagulant)[17] and blood compatibility assays were done.

Morphological studies on red blood cells (RBC) in whole blood

The morphological alteration study on RBCs by PAH capsule was done as per standardised method[18] with slight modification as described. In brief, human blood (50 µL) was incubated for 20 min at 37^{o}C with PAH nanocapsules (1.5 x 10^5, 3.0 x 10^5, 4.5 x 10^5 and 6.0 x 10^5). The diluted blood incubated with saline and silica nanoparticles (200µg/mL) was used as negative and positive controls respectively. RBCs morphology was examined by inverted microscopy using wet mounted slides at 40x magnification.

Isolation of monocytes from human blood and its morphological alteration study

Monocytes were isolated as previously described [17] (Supplementary information S1). 100 µl of the resuspended cells in PBS was incubated with PAH nanocapsule for 20 min at 37^{o}C. Cells incubated with saline and silica nanoparticles (200µg/mL) were used as negative and positive control respectively. Morphological changes were examined on wet mounted slides and the images captured by phase contrast microscopy (Leica DM IL, Germany) at 40x.

Haemolysis study

Haemolysis assay was done with varying concentration of PAH nanocapsules as per the reported method (Supplementary information S2).[19] Saline was used as negative control and silica nanoparticles (200µg/mL) as positive control.

Isolation of rat peripheral blood mononuclear cells (PBMCs) and cell culture

Peripheral blood mononuclear cells were isolated from rat, 3 ml volume of Histopaque 1083 solution was placed in a 15 ml tube and 3 ml blood was layered on top of this density gradient. After the centrifugation (400×g for 30 min at room temperature). The plasma layer was removed and discarded from the buffy coat, the PBMCs were carefully taken off by aspiration and washed with phosphate buffered saline (PBS) as described. [20] The isolated cells were cultured in collagen I coated plates and maintained at 37^0 C with 5% CO_2 in RPMI as cultured medium supplemented with 10% heat inactivated FBS, 1% l-glutamine 1% HEPES and 0.5% penicillin–streptomycin. The cells were dispersed in culture plates (1 x10^4

cells per well) and incubated with different concentrations of PAH nanocapsules for 24hr at identical environment.

Cytotoxicity studies

The cells were dispersed in 96 well plates (1 x 10^5 cells per well) and incubated with different concentrations of PAH nanocapsules (1.5 x 10^5, 3.0 x 10^5, 4.5 x 10^5 and 6.0 x 10^5) for 24, 48 and 72 h under identical environment. Freshly synthesized PAH nanocapsules were immediately tested for cytotoxicity on PBMCs. After the treatment period, cytotoxicity was evaluated by Trypan blue dye exclusion, [21] LDH assay, [22] MTT assay, [23] and Neutral red uptake cell viability assay.[24] (Supplementary information S3)

Genotoxicity study by comet assay

Comet assay was performed as previously reported. [25] Approximately 3×10^4 PBMCs cells were plated into 24-well tissue culture plates and incubated for 24 h in the absence or presence of PAH nanocapsules (1.5 x 10^5, 3.0 x 10^5, 4.5 x 10^5 and 6.0 x 10^5). The cells were then detached from the plate and processed in the slides and finally analyzed under a fluorescence microscope for the presence of comet tails.

ROS generation and lipid peroxidation in PBMCs

The estimation of ROS generation in PBMCs was done as previously reported [26] and described in Supplementary information S4. Lipid peroxidation level in PBMCs was measured as TBARS (thiobarbituric acid reactive substances).[27] (See Supplementary information S5)

Intracellular glutathione (GSH) depletion assay

The concentration of intracellular GSH was determined using colorimetric assay.[28] Briefly, cells were seeded in six-well plates at density of 5 $\times10^5$cells/well. After the treatments with 2mL of MSN for 24 h exposure, the medium was aspired and the cells were washed once with phosphate buffer saline (PBS). Then, the cells were scraped and centrifuged at 1100 rpm for 3min at 25°C; the supernatant was removed by aspiration. The cell pellets were resuspended in ice-cold metaphosphoric acid (MPA) and immediately homogenized and then centrifuged at 3000 rpm, 4°C for 10 min. Subsequently, the samples were mixed with 4-Chloro-1-Methyl-7-Trifluromethyl-Quinolinium ethylsulfate and 30% sodium hydroxide reagents were incubated for 10 min at room temperature in dark. The absorbances were measured spectrophotometrically at 400 nm.

Animal experiments

Sprague dawely rats were obtained from Department of Biochemistry, University of Kerala, India for specific study. All ethical guidelines were followed for the conduct of animal experiments in strict compliance with the Institutional animal ethical committee and committee for the purpose of control and supervision of experiments on animals (CPCSEA) government of India and ethical sanction no.IAEU-KU-24/2011-12-BC.AA (22) for the conduction of animal experiment.

***In vivo* Studies**

Experimental design

Sprague dawely male rats were randomly divided into 6 groups of 5 animals each and experimental period up to 30 days. Group-I Control, Group-II Saline treated (4ml/kg body weight), Group -III- PAH nanocapsules treated with (1.5 x 10^{12} PAH nanocapsules /Kg body weight), Group -IV- PAH nanocapsules treated with (3.0 x 10^{12}PAH nanocapsules /Kg body weight), Group-V- PAH nanocapsules treated with (4.5 x 10^{12} PAH nanocapsules /Kg body weight). Group-VI- Silica particle (177.5 mg/kg body weight). *In vivo* toxicity was also investigate by intravenous administration of nanocapsules by tail vein injection in Sprague dawely rats.

Inflammatory markers response study by PAH nanocapsules interaction

Activity of inflammatory markers in PBMCs namely, cyclooxygenase (COX), [29] lipooxygenase (LOX) [30] and nitric oxide synthase (NOS) [31] was investigated as described in Supplementary information S6

Enzyme linked immunosorbent assay (ELISA)

The release of PGE2 (prostaglandin E2) in the supernatants of PBMCs of different groups was measured as per the method of EIA kit (Cayman chemicals, USA).

Hematological study *in vivo*

Hematological parameters such as haemoglobin content, total white blood cell (WBC) count, RBC count, neutrophil, lymphocyte and blood urea nitrogen of the normal and nanocapsule injected rats were analysed using a semi-auto analyzer.

Toxicity markers status *in vivo*

Activity of the following toxicity markers- serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT),[32]; serum creatine kinase (CK); [33] and serum alkaline phosphatase (ALP) [34] were assayed using established procedure and described in Supplementary information S7

Gene level toxicity study by RT-PCR analysis

mRNA was isolated from various tissues like liver, aorta, heart, kidney, spleen and lungs for the study of important toxicity predictor genes like of interleukin1beta (IL-1β), monocyte chemotactic protein-1(MCP-1), transforming growth factor beta 1 (TGF- β1), kidney injury molecule-1(Kim-1), tumor necrosis factor alpha (TNF-α) and heat shock gene (Hsp70-1) by means of trizol reagent from Medox Biotech India PVT, Ltd. RT-PCR and PCR amplification were carried out using the RT-PCR kit from QIAGEN, India. Initial PCR activation for 15 min at 95^0C, followed by 3 steps of cycling process. Each cycle consists of denaturation for1min at 94^0C, annealing for 1min at 63^0C, extension for 1min at 72^0C, repeated for 37 cycles and final extension for 10min at 72^0C. The PCR products were run on 0.8% agarose gels and stained with ethidium bromide and visualized with a UV-transilluminator.

Western blotting studies of immunoregulatory proteins

The western blotting of cytokines (IL-8), chemokines (MIP-2) and cell adhesion molecules (VCAM-1 and ICAM-1) was done as reported.[35] Briefly, peripheral blood mononuclear cells were washed 3 times in PBS before lysis on ice in 150 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L NaN3, 10 mmol/L Tris (pH 8.0), 1 mmol/L PMSF, 5 mmol/L iodoacetamide, and 1% NP-40. Lysates were centrifuged at 15 000g for 10 minutes to remove debris and stored in aliquots at −20°C. Lysates were boiled for 5 minutes in nonreducing sample buffer (10 mmol/L Tris [pH 6.8], 2% SDS, 20% glycerol, and 0.001% [wt/vol] bromophenol blue) and resolved by 8% SDS–polyacrylamide gel electrophoresis (PAGE) with protein lysate from an equal number of cells loaded per lane. Separated proteins were transferred to nitrocellulose membranes and blocked for 1 hour at room temperature in PBS plus 3% (wt/vol) powdered milk and 0.1% Tween 20. Primary antibody of IL-8, MIP-2, VCAM-1, ICAM-1 (Abcam India) was added at the indicated dilution in blocking buffer and incubated on a rocking platform for 1 hour at room temperature. Binding was detected by incubation with peroxidase-conjugated secondary antibody (Sigma), diluted 1:1000 in blocking buffer and visualized by chemiluminescense.

Histological analysis of rat tissue

Tissues (liver, aorta, heart, kidney, lungs and spleen) were fixed with Bouin's fluid and processed for sectioning following standard techniques as described previously.[36] Tissues were embedded in paraffin wax (58-60°C), sectioned at 5-6μm thickness on a microtome (York Inc. USA) and stained with Harris' Hematoxylin-Aqueous Eosin stain and observed for histological changes under light microscope.

Statistical analysis

All statistical calculations were carried out with the statistical package for social sciences (SPSS) software program. The values are expressed as the mean ± SD. The data were statistically analyzed using analysis of variance (ANOVA) and significant difference of means was determined using Duncan's multiple range tests at the level of $p<0.05$.

Results and Discussion

PAH nanocapsules of 360nm size were characterised successfully by SEM (scanning electron microscope) (Fig.1A). Cationic nanomaterials often induce toxicity to blood cells and can participate in non-specific interactions with blood components which ultimately lead to severe inflammatory reactions or enhance fast clearance from the whole body system.[17] In order to access the hemocompatibility of PAH nanocapsule in human blood, haemolysis [37] and morphological change in RBCs were performed. Further, the effect of different concentration of PAH nanocapsules on haemolytic activity was studied as illustrated in Fig. 1B. It is observed that the haemolytic activity was lower than 2% which clearly suggests that these capsules are highly blood compatible. We note that positive control sample (silica nanoparticles) has produced significantly high level of haemolysis and it is has been reported that up to 5% hemolysis is permissible for biomaterials.[38] The level of ROS are reported to be increased on toxicity but in the PAH treated group there is an increase in the level of ROS (Fig 1C) showing the biocompatibility of PAH nanocapsules. Further, morphology of RBCs (Fig 1D) and monocytes (Fig 2B) didn't show any change in the morphology when compared with the control samples. Our results suggest that there is lesser or no aggregation of nanocapsules, which can change the morphology of cells and induce their lysis (Fig.1D). Thus, the blood compatibility studies demonstrated promising haemocompatibility of PAH nanocapsules.

Examination of inherent cytotoxicity of delivery vehicles is imperative so as to design biocompatible materials. To this end we have performed variety of cytotoxicty assays (e.g. MTT, Trypan blue, neural red and LDH) with rat PBMCs for different treatment durations- 24, 48 and 72hrs (Fig 3). MTT assay (Fig 3A) and Trypan blue assay (Fig 3D) clearly indicate the non-toxic nature of PAH nanocapsules to PBMCs even at higher concentration (6.0×10^5) for a period of 72 hrs. The MTT assay is used to measure mitochondrial activity, which is directly correlated to cell viability for both attached and poorly attached cells.[24] PAH nanocapsule showed no significant dose-dependent cytotoxic effects at the tested concentrations (1.5×10^5 to 6.0×10^5ml). More than 90% of the cells were viable when

compared to the control. The neutral red cell viability assay (Fig 3B) provides a quantitative estimation of the number of viable cells in a culture condition.[39, 40] It is based on the ability of viable cells to incorporate and bind neutral red, which predominantly accumulates in the lysosomes. The results showed above 90% cell viability at the highest tested concentration (6.0×10^5 and at longest treatment duration (72 hrs). Results from these assays indicate that there is no mitochondrial membrane damage or lysosomal leakage during treatment. Thus, these results further confirm the non-toxic nature of PAH nanocapsules. In another cell cytotoxicity assay, the proportion of lactate dehydrogenase (Fig 3C) found in the cell medium (relative to the value obtained from control cells) was similar at different treatment concentrations of PAH nanocapsules and for different treatment durations (24, 48 and 72 h). These results based on different cytotoxicity assays highlight the promising biocompatibility of the PAH nanocapsules. Moreover, these results were in agreement with previous reports.[41, 42]

Further, we performed comet assay (Fig 2C) with the polymer capsules to assess their genotoxicity profile. Genotoxicity studies indicate damage to the DNA and are essential for investigating the biological suitability of delivery systems.[43] Fig 2C clearly suggests that there is no formation of comet in the cells treated with different concentration of PAH capsules. However, the positive control samples of silica nanoparticles show the formation of comet which matches with the previous reports. This indicates that the capsules do not have any DNA level toxicity. The levels of ROS and thiobarbituric acid reactive substances (TBARS) are reported to be increased upon toxicity, but on administration of PAH capsules no toxicological responses was observed, demonstrating their cytocompatibility. Similarly, alteration in the levels of GSH content is a sign for oxidative stress mediated toxicity. In the present study (Fig.2D), the level of GSH in PAH treated cells was normal and similar to the GSH level observed in the control group. However, in the silica nanoparticles administered group there is a gradual reduction in the levels of GSH. These results demonstrate the non-toxic behaviour of PAH nanocapsules and are in agreement with earlier studies.[28]

Blood biochemical parameters such as serum glutamic oxaloacetic transaminase (SGOT) (Fig 4D), serum glutamic pyruvic transaminase (SGPT) (Fig 4A), creatine kinase (CK) (Fig 4C) and alkaline phosphatase (ALP) (Fig 4F) were assayed in the serum. Glutamic oxaloacetic transaminase (GOT) (Fig 4E) and glutamic pyruvic transaminase (GPT) (Fig 4B) were assayed in liver and kidney respectively. Aminotransferases such as SGOT and SGPT are useful in monitoring renal toxicity and hepatic toxicity induced by chemicals or stress

conditions. Serum aminotransferase levels will enhance when there is an inflammation in the liver and kidney. GOT and GPT are aminotransferases made almost exclusively by the liver cells. When the liver is inflamed it can leak GOT and GPT into the serum where it can be measured (SGOT and SGPT) as an indicator of inflammation. The levels of alkaline phosphatase (Fig 4F) and CK (Fig 4C) were found to be normal when compared with control. The present study showed no significant changes in the levels of these enzyme parameters in blood, liver or kidney during the entire treatment period, indicating the nontoxic behaviour of PAH nanocapsules in the animal system.[45] The biochemical marker status were normalised in PAH treated (Group III-V) in comparison with Group I and Group II. Our results are in agreement with other reports regarding toxicity. [16]

The inflammatory marker cyclooxygenase (COX) is the key enzyme catalyzing the conversion of arachidonic acid to prostaglandin H2, which is a precursor of wide variety of biologically active mediators such as PGE2, prostacyclins and thromboxane A2. Overproduction of these inflammatory mediators can lead to the inflammatory diseases.[45] The 5-lipoxygenase (5-LOX) pathway is responsible for the production of leukotrienes; inflammatory lipid mediators that have a role in the innate immunity but that can also play a proatherogenic role.[42] Nitric oxide production is increased by the inducible NOS (iNOS), subsequently brings about cytotoxicity and tissue damage.[46] The PAH treated group showed least immune response, showing that PAH nanocapsules didn't elicit any immune response by activating COX (Fig 5A), LOX (Fig 5B), NOS (Fig 5C) and PGE2 (Fig 5D) with different concentration in PBMCs

In vivo haematological parameters are very important for predicting nanocapsules toxicity in rat model. In the present work, interaction of polymer capsules with the blood components of rat have been investigated in detail by various haematological parameters such as haemoglobin content (Fig 6A), total WBC count (Fig 6B), RBCs count (Fig 6C), neutrophil (Fig 6D), lymphocyte (Fig 6E) and blood urea nitrogen (Fig 6F) by using a semi-auto analyzer and found to be within the normal range. Fig 6 clearly indicates that PAH capsules exhibit a nontoxic and biocompatible in *vivo* profile after 30 days of treatment in PAH treated groups (Group III- V) in comparison to Group I and Group II.

Interleukin-1 beta (IL-1β) is one of the toxicity markers of liver toxicity and has been shown to play an important role in IL-1 β signaling in acute and chronic liver injury.[47-50] The gene level expression of IL-1β was found to be normal in PAH treated groups (Group III-V; with different concentration of PAH nanocapsules) when compared with Group I and Group II

(Fig 7A). Monocyte chemotactic protein-1 (MCP-1) stimulates the migration of monocytic cells and predominantly activates polymorphonuclear leukocytes and results in severe inflammatory responses.[51] The expression of MCP-1 in aortic tissue was found to be normal in PAH nanocapsules treated groups (Group III-V; with different concentration of PAH nanocapsules) (Fig 7D) showing suitability of using PAH nanocapsules *in vivo*. Transforming growth factor-β1 (TGF-β1) is a locally generated cytokine that has been implicated as a major stimulator of tissue fibro inflammatory changes. TGF-β1 has a major influence on fibroblast proliferation and extracellular matrix production, particularly of collagen and fibronectin, while reducing degradation of these components.[52] Moreover, TGF-β1 gene up regulation in heart tissue leads to cardiotoxicity and apoptosis.[53] The level of TGF-β1 was found to be unaltered in PAH treated group as similar to Group I and II (Fig 7C).

Kidney injury molecule-1 (KIM-1) is an emerging biomarker and its expression and release are induced upon injury. Expression of KIM-1 is also associated with tubulointerstitial inflammation and fibrosis.[54] In the current study, the expression of KIM-1 level was found to be within the normal level (Fig 7B) showing that PAH nanocapsules are non toxic. The expression of tumor necrosis factor alpha (TNF-α) is found be an important marker for the activation of inflammation in spleen. TNF-α activates diverse signalling cascades leading to number of cellular responses, which include cell death, survival, differentiation, proliferation and migration. Vascular endothelial cells respond to TNF-α by undergoing a number of pro-inflammatory changes, which increase leukocyte adhesion, transendothelial migration and vascular leak and promote thrombosis.[55] The gene level expression of TNF-α was found to be normal in PAH treated groups (Group III-V) with different concentration of PAH nanocapsules) when compared with Group I and Group II (Fig 7E), thereby showing suitability for *in vivo* drug delivery applications. Hsp70-1 is one of the reliable markers for lung toxicity and is elevated under a variety of stressful conditions.[56] The expression of the Hsp70-1 gene was in the normal level for PAH treated groups when compared with the control group, but the silica treated group showed an altered level of gene expression (Fig 7F). Over all, the gene level study by RT PCR analysis (Fig 7) indicates the non-toxic nature of PAH nanocapsules.

The expression of immunomodulatory proteins are important markers of immunotoxicity.[57] Deregulation of cytokine gene expression explicitly represents alteration in the immune system. Consequently, analysis of cytokine gene expression has been widely employed for immunotoxicity testing i.e. evaluating the toxic effects of chemicals/delivery vehicles on the

immune system.[58-60] The levels of cytokines (IL-8) were highly elevated in silica nanoparticles treated group, while the PAH administered groups showed least level of activation of cytokines (Fig 8A). Chemokines are major regulators of the inflammatory response and play an important role in inducing toxicity. These chemokines participate in the chemotaxis and activation of neutrophils and macrophages respectively.[61] In the present study, there is an elevated expression of chemokines in silica treated group (Fig 8B), which is preceded by an increase in the transcription of the corresponding chemokine gene.[61] Inflammatory and immune responses involve a close contact between different populations of cells. Cell adhesion molecules are critical participants in the vascular dysfunction and tissue injury, which is associated with a wide variety of inflammatory and cardiovascular diseases.[62] These adhesive interactions mediate migration of cells to sites of inflammation and the effector functions of cells within the lesions.[63]Investigation of expression of cell adhesion molecules in case of different treatment groups showed an altered level of expression in case of silica nanoparticles treated groups. However, the PAH treated group showed least level of alteration of cell adhesion molecules (Fig 8C). Along with the gene expression studies, the immunoregulatory studies by western blotting also indicate that the PAH nanocapsules do not induce any immunotoxicological response.

Microscopic observation of various tissues by histopathological study also suggest the nontoxic nature of PAH nanocapsules. As observed in Fig 9-11, there is no sign of tissue damage or infiltration in case of the PAH nanocapsules treated groups. The morphological architecture of liver, aorta, lungs, heart, kidney and spleen showed almost same morphological similarity with Group I and Group II after 30 days of experimental period. Moreover, the histopathological observations are in agreement with earlier studies on toxicity of nanocapsules.[16, 64] Thus histopathological observation suggest nontoxic *in vivo* behaviour of PAH nanocapsules indicating their potential for *in vivo* drug delivery applications.

Conclusion

PAH nanocapsules were subjected to comprehensive investigation of toxicity and biocompatibility by employing a set of *in vitro* and *in vivo* studies. *In vitro* haemobiocompatibility studies, cytotoxicity assays and immunomodulatory studies in rat PBMCs suggest that PAH nanocapsules are haemocompatible and nontoxic. *In vivo* toxicity markers activity, haematological parameters alteration, RT-PCR analysis of important genes and expression of immunomodulatory proteins for PAH nanocapsules treated animals indicate their biocompatible and non-toxic nature. Histopathological studies of important

tissues showed almost normal architecture after treatment using different concentration of PAH nanocapsules for treatment duration of 30 days. Overall, the present study suggests that PAH nanocapsules are non-toxic and exhibit a biocompatible behaviour both at the *in vitro* and *in vivo* level for using it as a carrier for atherosclerotic drug delivery and imaging applications.

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Conflict of interest statement

Authors declare that they have no conflict of interest

References

1. A. Diaspro, S. Krol, O. Cavalleri, D. Silvano and A. Gliozzi, IEEE. Trans. Nanobios., 2002, 1, 110–115.
2. K. Pavankumar, K. Hemanth, R. Niranjan, M. Chiranjeevi and P. Radhika, *BioImpacts.,* 2012, 2, 71–81.
3. E. Donath, G.B. Sukhorukov, F. Caruso, S.A. Davis and H. Mohwald, *Angew. Chem.,* 1998, 110, 2324–2327.
4. G.B. Sukhorukov, E. Donath, S. Davies, H. Lichtenfeld and F. Caruso, *Polym.Adv.Technol.,* 1998, 9, 759–767.
5. Y.Wang, V.Bansal, A.N. Zelikin and F. Caruso, *Nano. Lett.,* 2008, 8, 1741-1745.
6. A.J. Lusis, *Nature*., 2000, 407, 233–241.
7. S.E. Nissen, S.J. Nicholls, I. Sipahi, P. Libby, J.S. Raichlen, C.M. Ballantyne, J. Davignon, R. Erbel, J.C. Fruchart, J.C. Tardif, P. Schoenhagen, T. Crowe, V. Cain, K. Wolski, M. Goormastic and E.M. Tuzcu, *JAMA.,* 2006, 295, 1556–1565.
8. C.F. Nathan, *J. Clin. Invest*., 1987, 79, 319–326.
9. J.G. Dickhout, G.S. Hossain, L.M. Pozza, J. Zhou, S. Lhotak and R.C. Austin, *Arterioscler. Thromb.Vasc Biol*., 2005, 25, 2623–2629.
10. G. D. Jeffrey, B. Sana and C. A. Richard, *Arterioscler. Thromb. Vasc Biol*., 2008, 28, 1413-1415.
11. J. Zhou, S. Lhotak, B.A. Hilditch and R.C. Austin, *Circulation.,* 2005,111, 1814 - 1821.
12. G. Oberdorster, *J. Int. Med.,* 2010, 267, 89–105.

13. G. Oberdorster, E. Oberdorster and J. Oberdorster, *Environ. Health. Perspec*., 2005, 113 823–839.
14. N.A. Monteiro-Riviere, A.O. Inman and L.W. Zhang, *Toxicol. Appl. Pharmacol*., 2009, 234, 222–235.
15. A. Kroll, M.H. Pillukat, D. Hahn and J. Schnekenburger, *Eur. J. Pharm. Biopharm.,* 2009, 72 , 370–377.
16. R.P. Bulcao, F.A. de Freitas, E. Dallegrave, C.G. Venturini, M. Baierle, J. Durgante, E .Sauer, C. Cassini, C.T. Cerski, P. Zielinsky, M. Salvador, A.R. Pohlmann, S.S. Guterres and S.C. Garcia, *Eur. J. Pharm. Biopharm*., 2013, 00123–129.Pii: S0939–6411(13).
17. M.R. Rekha and C.P. Sharma, *Biomater.,* 2009, 34*,* 6655–6664.
18. L. He, L.Yang, Z.R. Zhang, T.Gong, L. Deng, Z. Gu and X. Sun, *J. Appl. Polym.Sci*., 2009, 5, 2933–2944.
19. J.M. Koziara, W.S. Akers, S.P. Ferraris and R.J. Mumper, *Pharm*. *Res*., 2005**,** 22**,** 1821–1828.
20. A. Radhika, S.S. Jacob and P.R. Sudhakaran, *Mol. Cell. Biochem.,* 2007, 5, 133–143.
21. R.I. Freshney, Culture of animal cells a manual of basic technique, Wiley-Liss, New York, 3rd edn., 1993, pp 496.
22. C. Legrand, J.M. Bour, C.Jacob, J.Capiaumont, A.Martial, A.Marc, M. Wudtke, G. Kretzmer, C. Demangel and D. Duval, *J Biotechnol.,* 1992, 3,231–423.
23. T. Mosmann, *J. Immunol. Methods.,* 1983, 65, 55–63.
24. A. Valdivieso-Garcia, R.C. Clarke, K. Rahn, A. Durette, D.L. Macleod and C.L. Gyles, *Appl. Environ. Microbiol.*, 1993, 6, 1981–1983.
25. Q. Mu, N.S. Hondow, L. Krzemiński, A.P. Brown, L.J. Jeuken and M.N. Routledge, *Part. Fibre. Toxicol.,* 2012, 23, 9–29.
26. H. Wang and J.A. Joseph , *Free Rad*. *Biol*. *Med*., 1999, 27**,** 612–616.
27. W.G.J. Niehaus and B. Samuelsson, *Eur. J. Biochem.,* 1968, 6:126–130.
28. U. DeSimone, L. Manzo, A. Profumo and T. Coccini, *J. Toxicol.,* 2013, 2013, 931785.
29. T. Shimizu, K. Kondo and O. Hayaishi, *Arch. Biochem. Biophys.,* 1981, 206, 271–276.
30. B.Axelrod, T.M. Cheesebrough and B. Laakso, Lipoxygenase from soybeans Methods in Enzymology. Academic Press, 1981, 71,441–453.

31. M. Salter, R.G. Knowles and S. Moncada, *FEBS. Lett.,* 1991, 291, 145–149.
32. S. Reitman and S. Frankel, *Am. J. Clin. Pathol.,* 1957, 1, 56–63.
33. S.B. Rosalki*, J. Lab. Clin. Med.,* 1967, 69, 696–705.
34. H. Varley, A.H. Gowenlock and H. Bell, Enzymes. In Practical Clinical Biochemistry. William Heinemann Med Books London, (William H Ed.), 1975, pp 813.
35. P.J. Gough, R. David. Greaves, S. Hiroshi, H. Tomi, O.H. Mikko, T. Mikko, Y.H. Seppo, K .Tatsuhiko and G. Siamon, *Arterioscler. Thromb. Vasc. Biol.*, 1999, 3, 461–471.
36. S.L. Lamberg and R. Rothstein, Laboratory Manual of Histology and Cytology. Westport CT, Avi Publishing,1978.
37. J.P. Singhal and A.R. Ray, *Biomater.*, 2002, 4, 1139–1145.
38. W. Paul and C.P. Sharma, *Trend. Biomater. Arti. Org.,* 2011, 25, 91–94.
39. M.L. Richard, I.R. Rivkah and C.G. Edward, *J. Invest. Dermato.,* 1992, 98, 725–729.
40. M. Vibin, R. Vinayakan, A .John, V. Raji, C.S. Rejiya, N.S. Vinesh and A. Abraham, *J. Nanopart. Res.,* 2011, 13, 2587–2596.
41. C.S. R, J. Kumar, V. Raji, M. Vibin and A. Abraham, *Pharmacol. Res.,* 2012, 65*,* 261–269.
42. V. Raji, K. Jatish , C.S. Rejiya, M. Vibin, J .Annie and A. Abraham, *J. Exp. Nanosci.*, 2012, 7, 174–188.
43. M. Naya, N. Kobayashi, M. Ema, S. Kasamoto, M. Fukumuro, S.Takami, M. Nakajima, M .Hayashi and J. Nakanishi, *Regul. Toxicol. Pharmacol.,* 2012, 62*,* 1–6.
44. R.C. Lasagna, R.D. Gonzalez, M.A. Barria, I .Olmedo, A. Clos, R.V.M. Sadagopa, A. Urayama, L. Vergara, M.J. Kogan and C. Soto, *Biochem. Biophys. Res. Commun.,* 2010, 393*,* 649–655
45. S.K. Kavitha, V. Viji, B. Shobha, K. Kripa, V. Shalini and A. Helen, *Cell. Immunol.,* 2012, 272, 283–289.
46. O, Radmark and B. Samuelsson, *Prostaglandins. Other. Lipid. Mediat.,* 2007, 83, 162–174.
47. R.G. Gieling, K. Wallace and Y.P. Han, *Am. J. Physiol. Gastrointest. Liver. Physiol.,* 2009, 296, 1324–1331.
48. A.B. Imaeda, A. Watanabe, M.A.Sohail, S. Mahmood, M. Mohamadnejad, F.S. Sutterwala, R.A. Flavell and W.Z. Mehal, *J. Clin. Invest,* 2009, 119, 305–314.

529 49. K. Miura, Y. Kodama, S. Inokuchi, B. Schnabl, T. Aoyama, H. Ohnishi, J.M. Olefsky,
530 D.A. Brenner and E. Seki, *Gastroentero.,* 2010,139, 323–334.
531 50. J. Petrasek, A. Dolganiuc, T. Csak, E.A. Kurt-Jones, G. Szabo, *Gastroenterology.,*
532 2011, 140, 697–708.
533 51. T.J. Schall and K.B. Bacon, *Curr. Opin. Immunol.,* 1994, 6, 865–873.
534 52. W.A. Border and N.A. Noble, *N. Engl. J. Med.,* 1994,331, 1286–1292.
535 53. F. Kuwahara, H. Kai, K. Tokuda, M. Kai, A. Takeshita, K .Egashira and T. Imaizumi,
536 *Circu.,* 2002, 10,130–135.
537 54. A.I. Lim, S.C. Tang, K.N. Lai and J.C. Leung, *J. Cell. Physiol.,* 228 (2013) 917–924.
538 55. J.R. Bradley, TNF-mediated inflammatory disease, *J. Pathol.,* 2008, 214,149–160.
539 56. D. Wirth, E. Christians, C. Munaut, C. Dessy, J.M. Foidart and P. Gustin*, Cell. Stress.*
540 *Chaperones.,* 2002, 7, 387–395.
541 57. P.A. Janeesh, V. Sasikala, C.R. Dhanya and A. Abraham, *Int. Immunopharmacol.,*
542 2014, 18,191–197.
543 58. V.R. House and J. Descotes, Cytokines in Human Health: Immunotoxicology,
544 Pathology, and Therapeutic applications. Methods in pharmacology and toxicology
545 Humana press, USA, 2007.
546 59. D.B. Oliveira, K. Gillespie, K. Wolfreys, P.W. Mathieson, F. Qasim and J.W.
547 Coleman, *Eur. J. Immunol.,* 1995, 25, 2259–2264.
548 60. J. Dastych, D.A. Walczak, J. Wyczolkowska and D.D. Metcalfe, J. *Allergy. Clin.*
549 *Immunol.,* 1999, 103,1108–1114.
550 61. J.M. Queally, B.M. Devitt, J.S. Butler, A.P. Malizia, D. Murray and P.P. Doran, *J.*
551 *Orthop. Res.,* 2009, 7, 855–864.
552 62. C. Golias, E. Tsoutsi, A. Matziridis, P. Makridis, A. Batistatou and K.
553 Charalabopoulos, *In Vivo*., 2007, 5,757–769.
554 63. J.M. Crawford and K. Watanabe, *Crit. Rev. Oral. Biol. Med.,* 1994, 5, 91-123.
555 64. P.A. Janeesh, H. Sami, C.R. Dhanya, S. Sivakumar and A. Abraham, *Chem. Biol.*
556 *Drug. Des.*, 2014 doi: 10.1111/cbdd.12313.

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Figure legends

Fig. 1 [A] SEM micrograph of Polyallylamine Hydrochloride (PAH) Nanocapsules; [B] Haemolysis study in blood; [C] ROS generation peripheral blood mononuclear cells (PBMCs) ; [D] Morphological studies on Red blood cell (R.B.C) in whole blood. The cells were treated with silica nanoparticles 200 μg/mL; PAH nanocapsules with varying number (PAH 1-1.5 x 10^5 capsules/ml, PAH 2-3.0 x 10^5 capsules/ml, PAH 3- 4.5 x 10^5 capsules/ml and PAH 4- 6.0 x 10^5 capsules/ml). Each value represents mean ± SD of six values.

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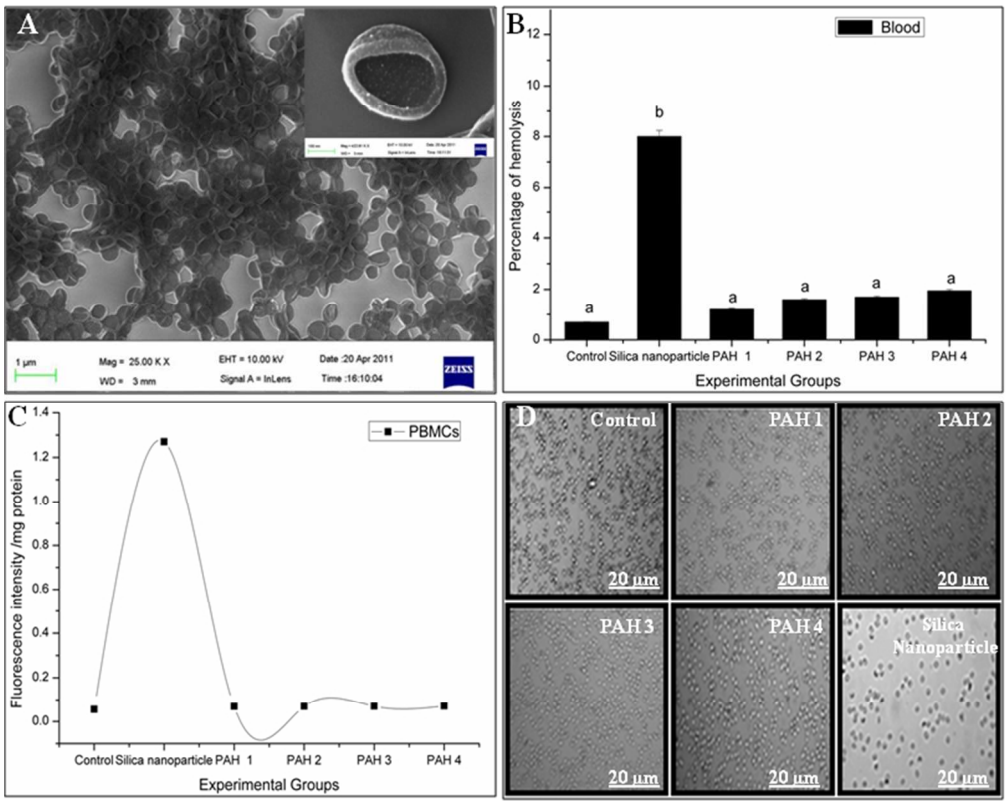

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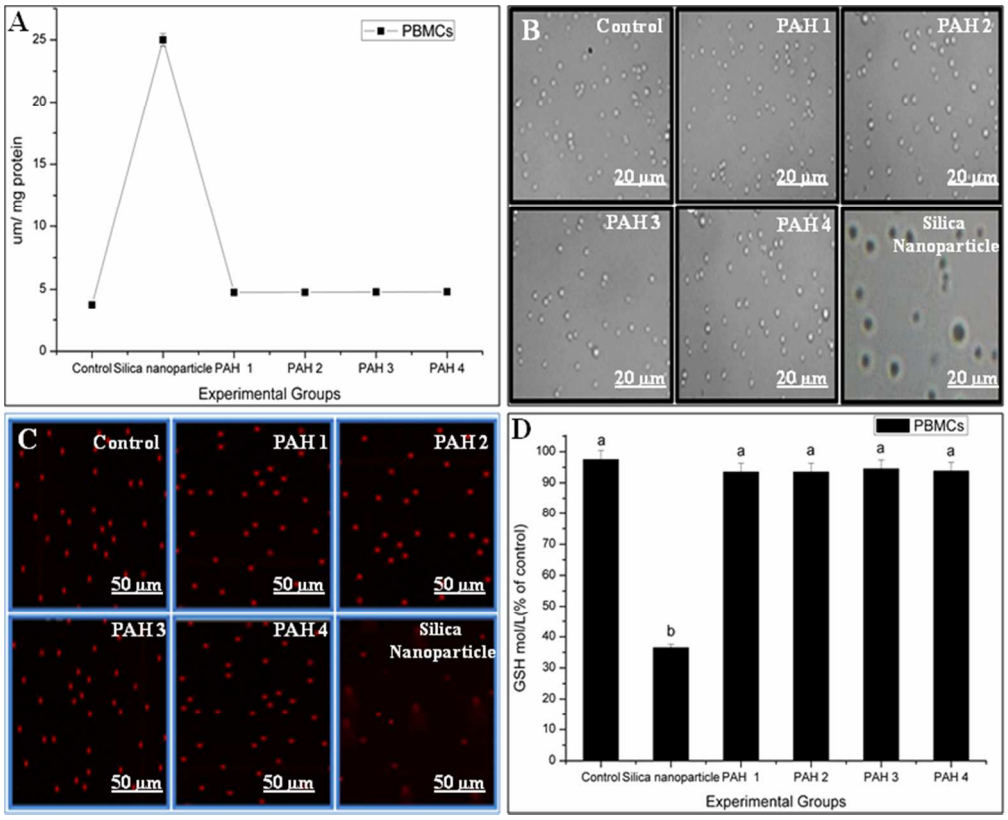

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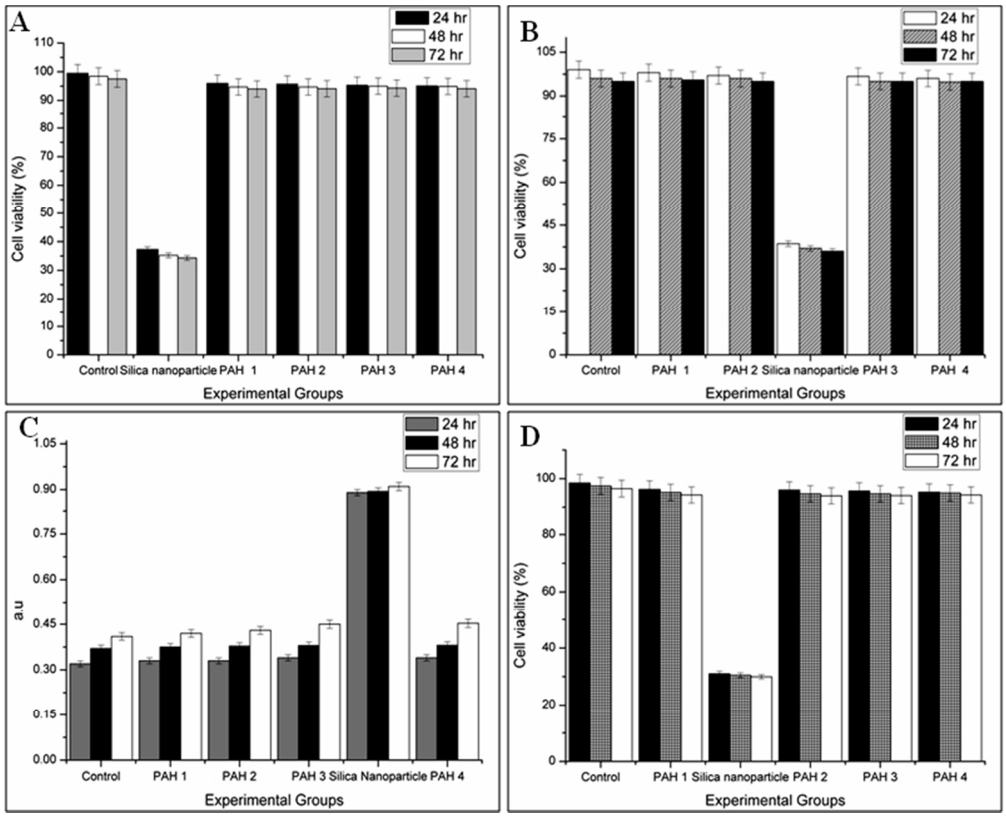

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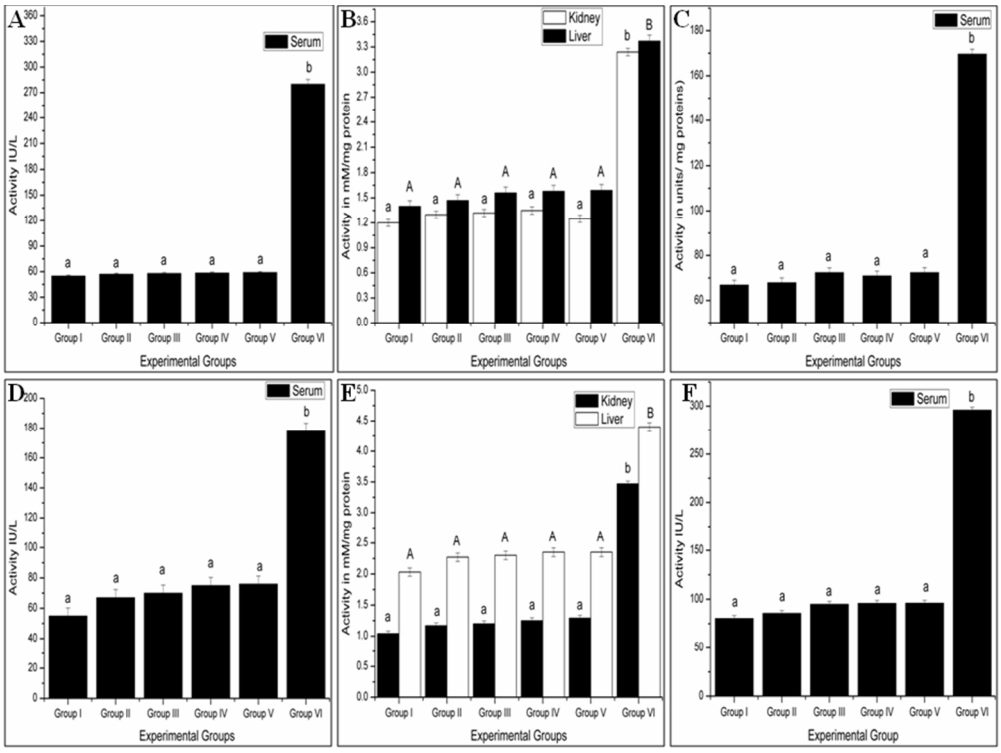

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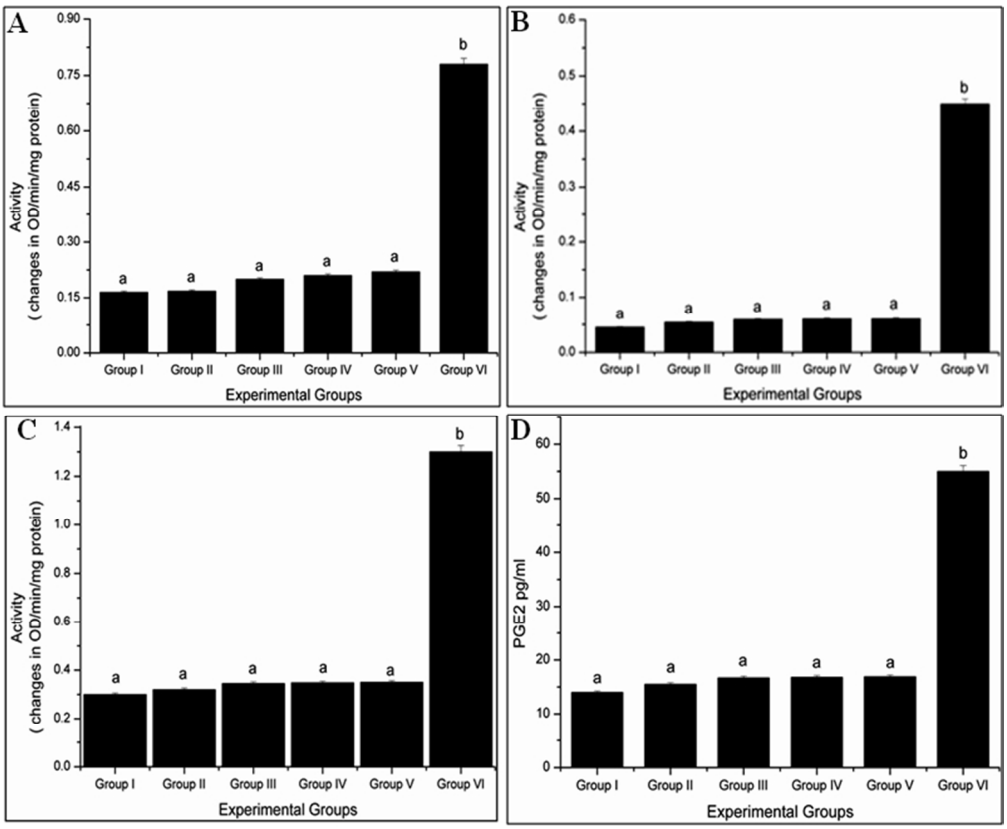

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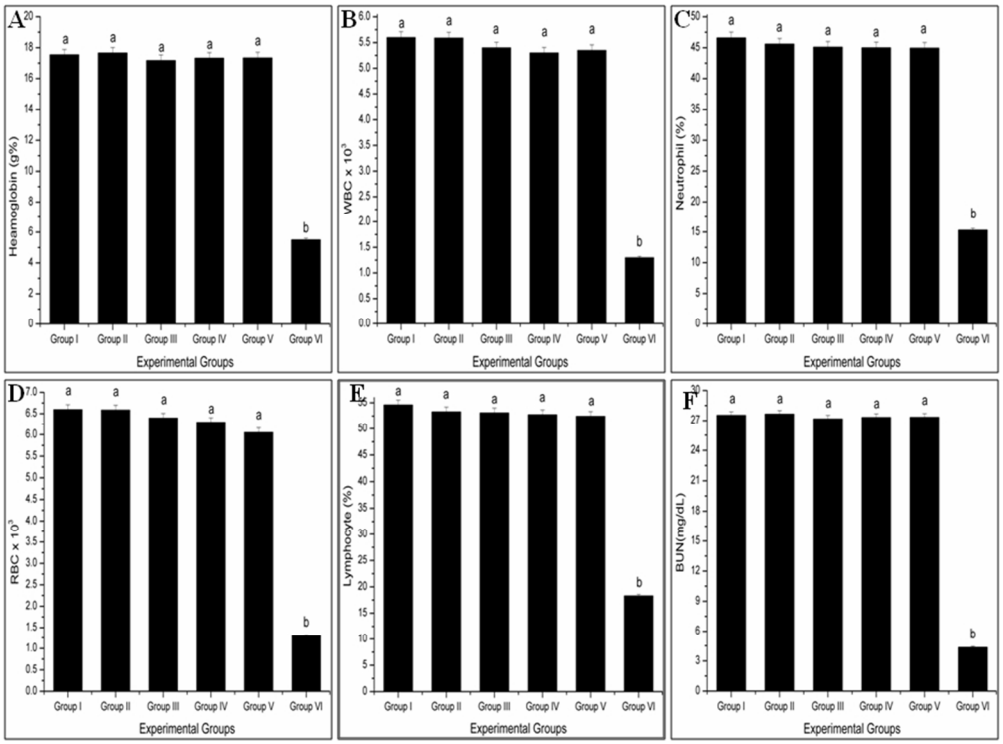

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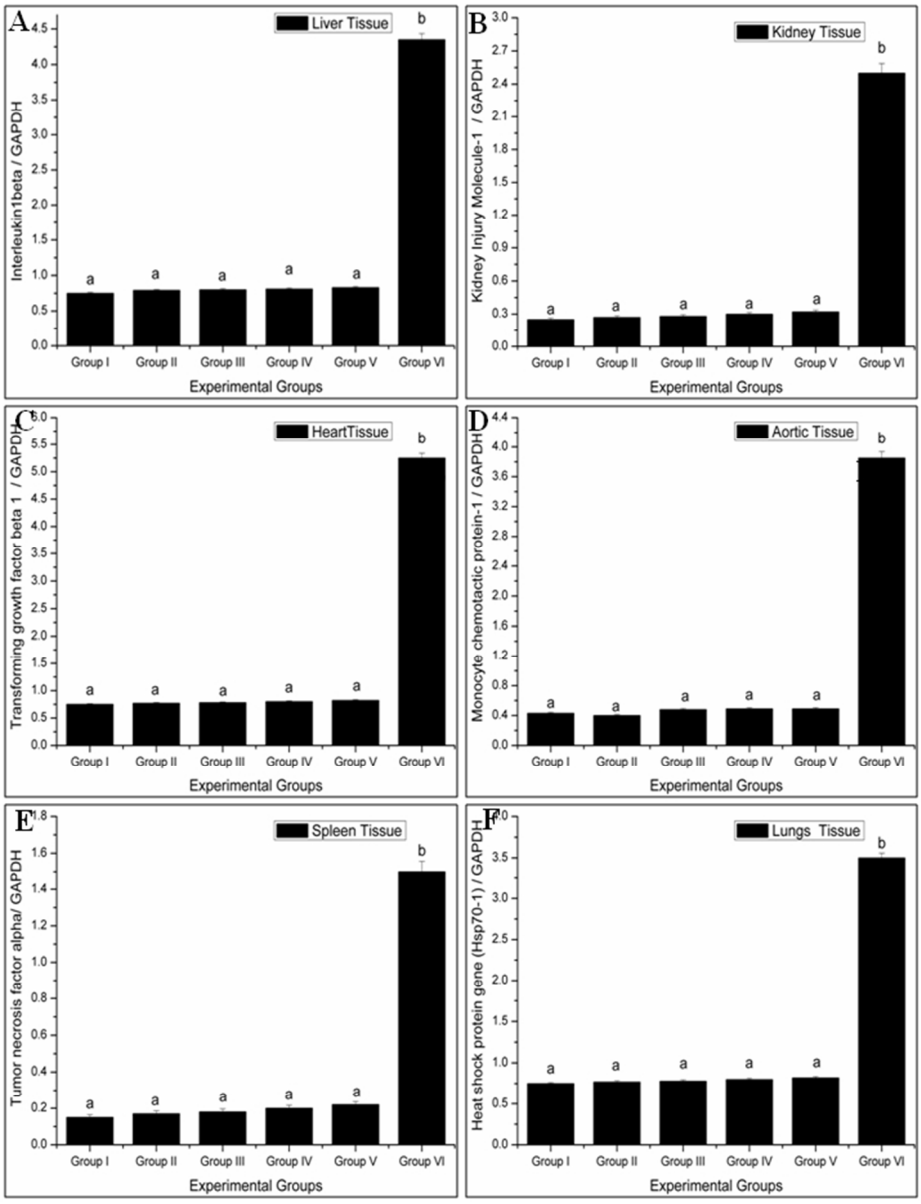

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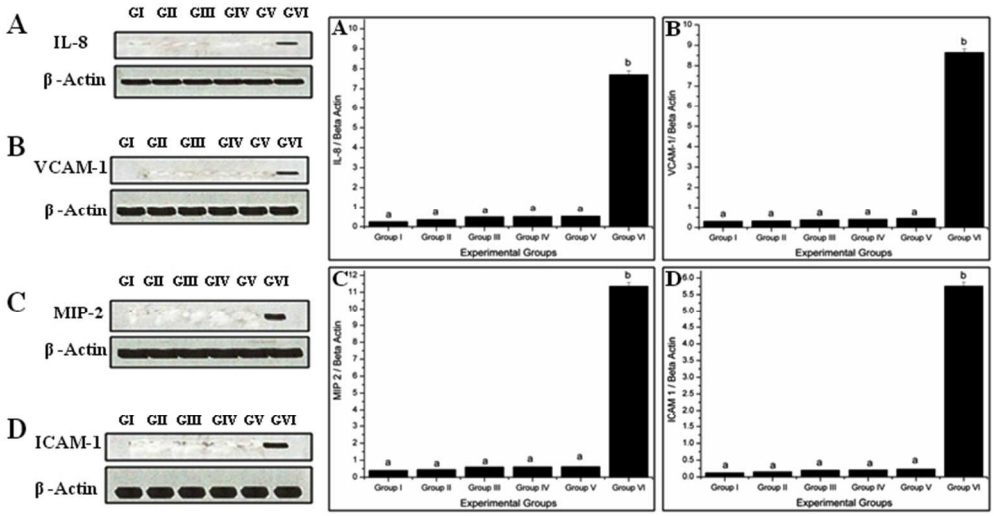

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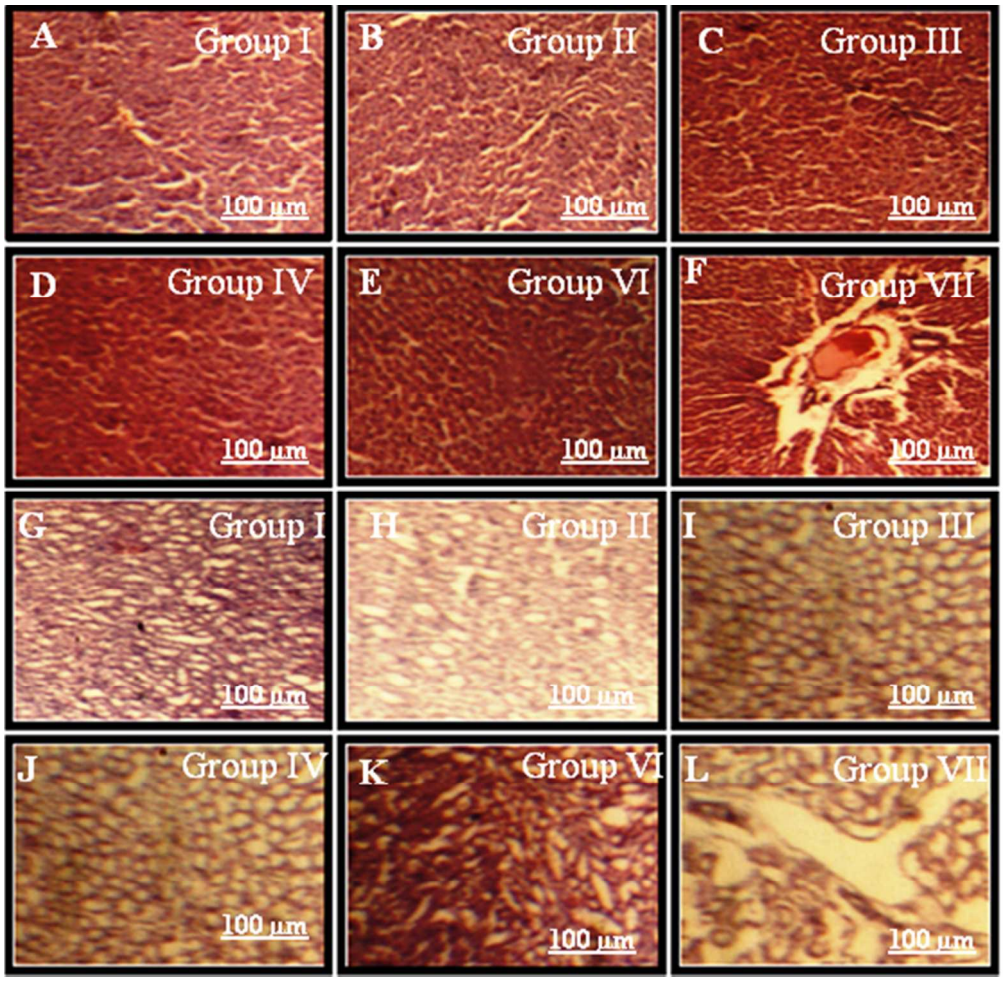

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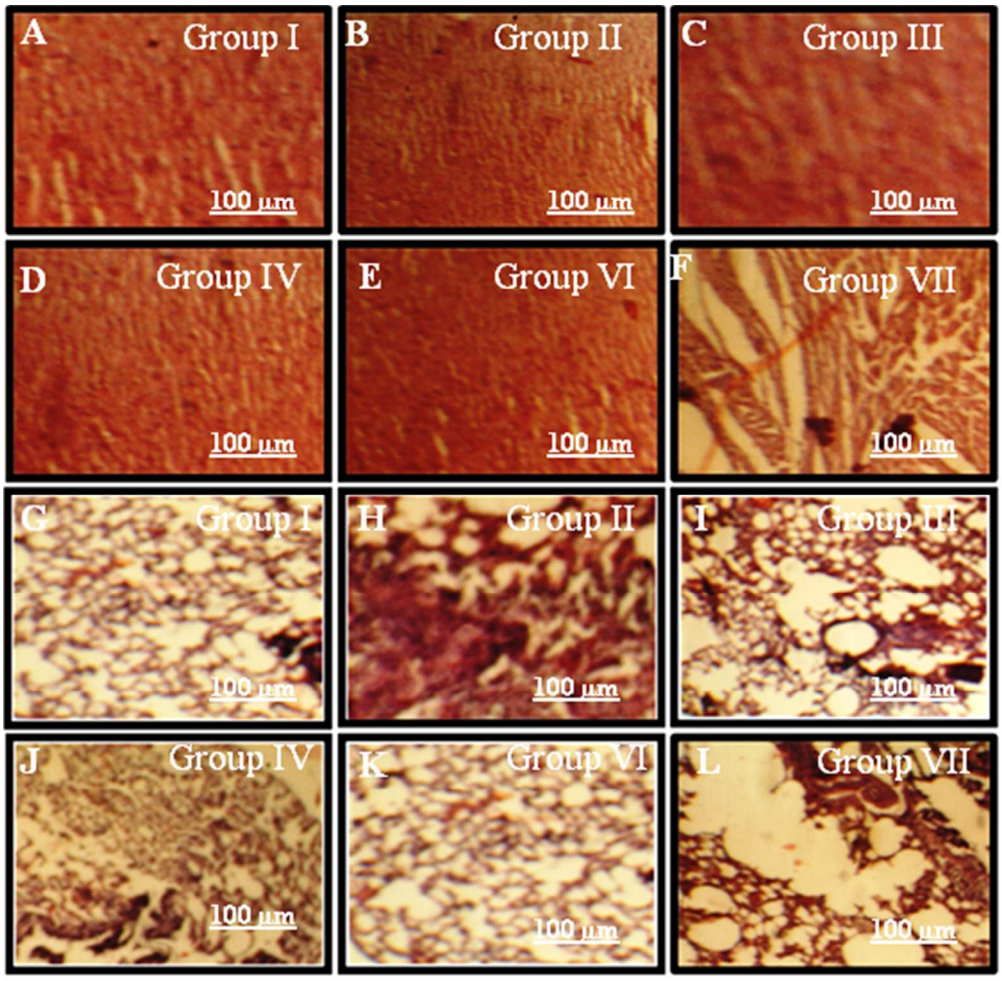

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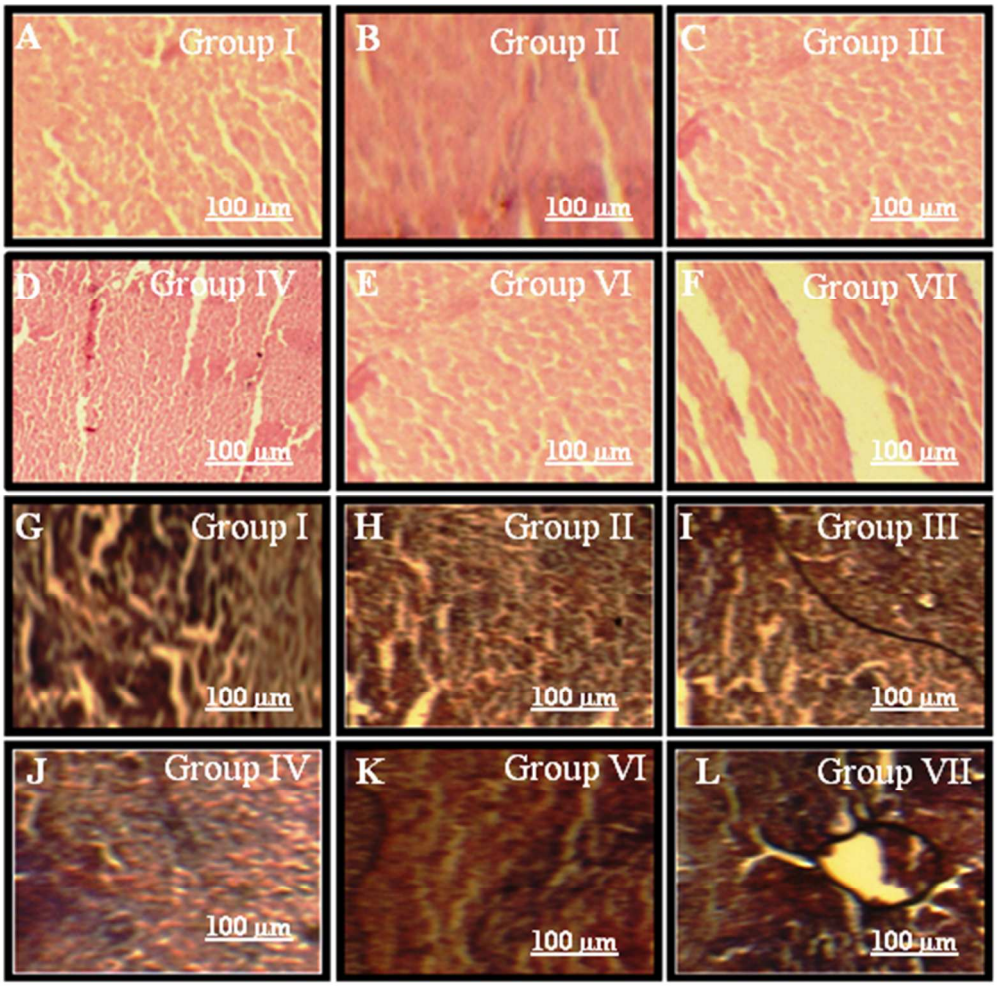

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Table of contents entry

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