

Published in final edited form as:

Artif Cells Blood Substit Immobil Biotechnol. 2008 ; 36(6): 513–524. doi:10.1080/10731190802554224.

Long-term Effects on the Histology and Function of Livers and Spleens in Rats after 33% Toploading of PEG-PLA-nano Artificial Red Blood Cells

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Abstract

This study is to investigate the long-term effects of nanodimension PEG-PLA artificial red blood cells containing hemoglobin and red blood cell enzymes on the liver and spleen after 1/3 blood volume top loading in rats. The experimental rats received one of the following infusions: Nano artificial red blood cells in Ringer lactate, Ringer lactate, stroma-free hemoglobin, polyhemoglobin, and autologous rat whole blood. Blood samples were taken before infusions and on days 1, 7, and 21 after infusions for analysis. Nano artificial red blood cells, polyhemoglobin, Ringer lactate and rat red blood cells did not have any significant adverse effects on alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatine kinase, amylase and creatine kinase. On the other hand, stroma-free hemoglobin induced significant adverse effects on liver as shown by elevation in alanine aminotransferase and aspartate aminotransferase throughout the 21 days. On day 21 after infusions rats were sacrificed and livers and spleens were excised for histological examination. Nano artificial red blood cells, polyhemoglobin, Ringer lactate and rat red blood cells did not cause any abnormalities in the microscopic histology of the livers and spleens. In the stroma-free hemoglobin group the livers showed accumulation of hemoglobin in central veins and sinusoids, and hepatic steatosis. In conclusion, injected nano artificial red blood cells can be efficiently metabolized and removed by the reticuloendothelial system, and do not have any biochemical or histological adverse effects on the livers or the spleens.

Keywords

Nanotechnology; nanomedicine; nanobiotechnology; PEG-PLA nanocapsules; artificial red blood cells; reticuloendothelial system; PolyHermoglobin

INTRODUCTION

The first artificial red blood cells (artificial RBCs) prepared by microencapsulation of hemolysate have oxygen dissociation curve similar to RBCs [1]. However, these artificial RBCs, even with diameters of down to one micron, survived for a short time in the circulation after intravenous injection [2]. Our study shows that the long circulation time of

RBCs is due to the presence of neuraminic acid on the membrane [3]. This led us to study the effects of changing surface properties of the artificial RBCs [2, 3]. This has resulted in significant increases in circulation time, but still not sufficient for clinical uses [3]. Later, other researchers were able to increase the circulation time further by preparing smaller lipid membrane Hb artificial cells with diameter of about 0.2 micron [4]. The incorporation of polyethylene glycol (PEG) onto the lipid membrane artificial RBCs resulted in useful increases in the circulation time [5]. Ongoing efforts are being carried out to develop this in preclinical studies [6]. We have used a biodegradable polymer, polylactic acid (PLA), for the microencapsulation of Hb, enzymes, and other biologically active material since 1976 [7]. More recently, we started to prepare nanodimension artificial RBCs of less than 100 nanometers mean diameter using PLA membrane and other biodegradable polymers [8–10]. We have replaced most of the 6 g/dl of lipid membrane in Hb lipid vesicles with 1.6 g/dl of biodegradable polymeric membrane material. This marked decrease in the lipid component would lessen effects on the RES and lessen lipid peroxidation in ischemia-reperfusion. Unlike lipid membrane, biodegradable polymeric membrane is permeable to glucose. Thus, the inclusion of RBC enzymes prevents MetHb formation, even at 37°C, and we can also convert MetHb to Hb at 37°C (11–13). In addition, unlike lipid membrane, the nanocapsule membrane allows plasma factors like ascorbic acid to enter the nanocapsules to prevent MetHb formation. In order to increase the circulation time, we synthesized a new PEG-PLA copolymer to prepare Nano artificial RBCs [11–13]. After extensive research, we now have a circulation time in rats that is double that of polyhemoglobin [11–13]. Our previous reports showed that the PEG-PLA artificial RBCs has no adverse effect on the renal function and systemic biochemistry [14]; in this report, we investigated the effect of 1/3 volume toploading infusions with PEG-PLA Nano artificial RBCs on the livers and spleens when followed on days 1, 7, and 21 after infusions.

MATERIALS AND METHODS

Preparation of Hb Solution

Stroma-free hemoglobin was prepared by the method as described [12]. Briefly, SFHb was obtained by hypotonic hemolysis of bovine RBCs and it was made stroma-free by toluene extraction and high-speed centrifugation. The resulting solution contained 10–15 g Hb/dl.

Preparation of PolyHb

This has been described in detail [13]. Briefly, 0.5 M glutaraldehyde was added and allowed to react for 24 hr at 4° C under anaerobic conditions with constant stirring. The reaction was stopped with excess 2.0 M lysine HCl at a molar ratio of 100:1 (lysine/Hb). Removal of reactants and free Hb molecules were carried out using dialysis and molecular exclusion chromatography [13].

Preparation of PEG-PLA Nano Artificial RBCs

This has been described in detail [13]. Briefly, PEG-PLA copolymer was prepared from 1.5 g of D,L-PLA[M.W. 15,000] and 0.75 g of methoxypolyethylene glycol [M.W. 2000] [13]. Just before use, 150 mg of the PEG-PLA copolymer were dissolved in 8 ml acetone. Fifty mg of hydrogenated soybean phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) were

dissolved in 4 ml ethanol. The two solutions were mixed and emulsified with the PolyHb solution to form Nano artificial RBCs. This could be followed by glutaraldehyde crosslinking to reduce the permeability of the membrane [13]. This was then followed by vacuum solvent extraction to remove the organic solvent and high porosity ultrafiltration to remove unencapsulated Hb or PolyHb [13].

Toploading

Twenty male Sprague-Dawley rats in the weight range of 300±40 g were used. They were randomly assigned into 5 groups. Each group received one of the following solutions: 1, PEG-PLA Nano artificial RBCs in Ringer lactates; 2, LactRing as control; 3, SFHb; 4, PolyHb; 5, Autologous rat RBCs.

Rats were anesthetized with sodium pentobarbitum (60 mg/kg), then unilateral femoral vein was cannulated using 3 cm length PE10 tubing (0.28 mm inner, 0.61 mm outer diameter, Clay Adams, Becton Dickinson, Sparks, MD, USA). Each rat received intravenous infusion of 33% of the total blood volume of one of the above 5 solutions. Total blood volume in ml was calculated as 6.5% of body weight in grams.

Histology Study

At day 21 after injection of various solutions, rats in all groups were sacrificed; the liver, spleen, and lung were excised for histology examination. The tissue specimen was fixed in 10% formalin solution, embedded in paraffin, then cut into serial 5-µm thick sections, mounted on slides, and then sections were stained with hematoxylin and eosin (H&E) for histology examination.

Blood Chemistry

Blood samples were taken from rats before injections, and on days 1, 7, 21 after injections. Plasma samples were stored in -76°C freezer until tested in the analytical laboratory of the McIntyre Animal Centre, McGill University. Blood alkaline phosphatase (AKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), total bilirubin (TBil), cholesterol (CH), amylase, and creatine kinase (CK) were determined.

Statistics

Blood chemistry assays were presented as mean±S.E. For multiple comparisons of biochemical data in all groups, we performed one-way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered significant.

RESULTS

Histology

There was no observable adverse effect on the microscopic histology of the liver receiving toploading of PEG-PLA Nano artificial RBCs, LactRing, PolyHb, or autologous RBCs. However, in the liver of the SFHb infused animals there is accumulation of Hb in the central vein and sinusoids, and steatosis (Figure 1). There were no microscopic abnormalities in the spleens of all groups (Figure 2).

Biochemistry

Figure 3 shows that 33% volume toploadings of Nano artificial RBCs, LactRing, SFHb, PolyHb, or RBC do not result in elevation of the systemic levels of total bilirubin in rats. One day after infusion of Nano artificial RBCs there was a transient increase of total bilirubin, but on day 7 and 21, the total bilirubin levels were lower than the other solutions. It should be noted that the circulation half time of PEG-PLA Nano artificial RBCs was 24 hours in rats (Figure 3). Since standard centrifugation did not remove PEG-PLA Nano artificial RBCs, both the nanoparticles and the Hb may have interfered with the measurement for total bilirubin on day 1. In rat the circulation half time of PolyHb after 30% toploading is 12 hours and that for SFHb is even less.

Figure 4 shows that the albumin level for the Nano artificial RBCs group remained the highest of all the solutions infused.

There was no elevation of the systemic cholesterol level for the groups receiving 33% volume toploading of Nano artificial RBCs, LactRing, SFHb, PolyHb or RBCs (Figure 5).

Figures 6, 7, and 8 show that there was no elevation in the systemic levels of CK, AST or ALT in rats infused with 33% volume of Nano artificial RBCs. There was also no significant elevation of systemic levels of CK, AST, and ALT in rats infused with 33% volume of LacRing or RBCs. There was an increase in ALT on day 21 for PolyHb. Infusion of SFHb resulted in a consistent increase in the systemic level throughout the 21 days for AST and ALT.

Infusion of 33% total blood volume of Nano artificial RBCs, LactRing, SFHb, PolyHb or RBC did not result in any significant increase in systemic amylase when compared to the control level before infusion (Figure 9). AKP in rats infused with 33% volume of LactRing, SFHb, PolyHb, or RBC did not result in any significant changes throughout the 21 days. Infusion of Nano artificial RBCs resulted in an increase of AKP on day 7 (Figure 10 upper) however, when this was repeated, the results did not show a significant increase (Figure 10 lower).

DISCUSSION

In our present study we observed that SFHb caused liver function damage. The chemical cross-linking of hemoglobin with glutaraldehyde into poly-hemoglobin when infused did not show damage to liver and spleen. Our results also showed that PEG-PLA Nano artificial RBCs containing Hb did not induce liver function disorders, and there were no histological abnormalities in the livers and spleens.

Our present results showed that the PEG-PLA Nano artificial RBCs can be sufficiently captured by RES and metabolized when systemically infused, and PEG-PLA Nano artificial RBCs artificial red blood cells do not have adverse effects on the RES. The PEG-PLA Nano artificial RBCs are very small so that they may effectively prevent embolization of capillaries and venules in microcirculation. Further studies will be carried out using electromicroscopic analysis and study of effects on phagocytic activities.

Acknowledgments

This research was supported by the Canadian Institutes of Health Research and the Quebec Ministry of Health's Hemovigilance Program in the form of a FRSQ Research Group (d'équip) on Blood Substitutes in Transfusion Medicine.

Abbreviations

Hb	hemoglobin
MetHb	methemoglobin
NanoRBC	nanodimension artificial red blood cells
PEG	polyethylene glycol
PLA	polylactide
PolyHb	polyhemoglobin
RBC	red blood cells
SFHb	stroma-free hemoglobin
RES	reticuloendothelial system
AKP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CK	creatine kinase
TBil	total bilirumin
CH	cholesterol

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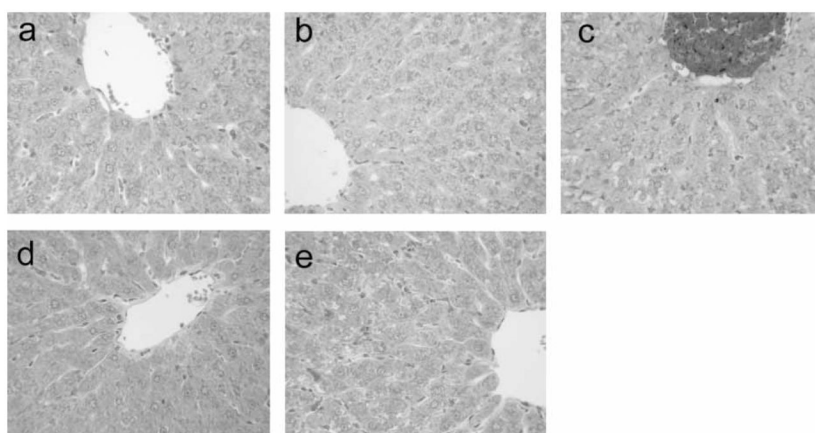


Figure 1. Histology of liver in rats toploading with NanoRBC (a), LactRing (b), SFHb (c), PolyHb (d), or RBC (e). Under light microscope, there was no abnormality in groups a, b, d, and e. For group c infused with SFHb there is Hb accumulation and steatosis. H&E staining, $\times 400$.

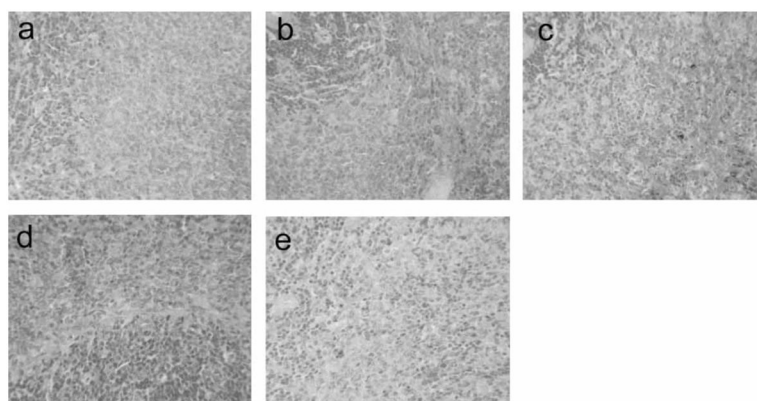


Figure 2. Histology of spleen in rats toploading with NanoRBC (a), LactRing (b), SFHb (c), PolyHb (d), or RBC (e). No obvious abnormalities were seen in all groups. H&E staining, $\times 400$.

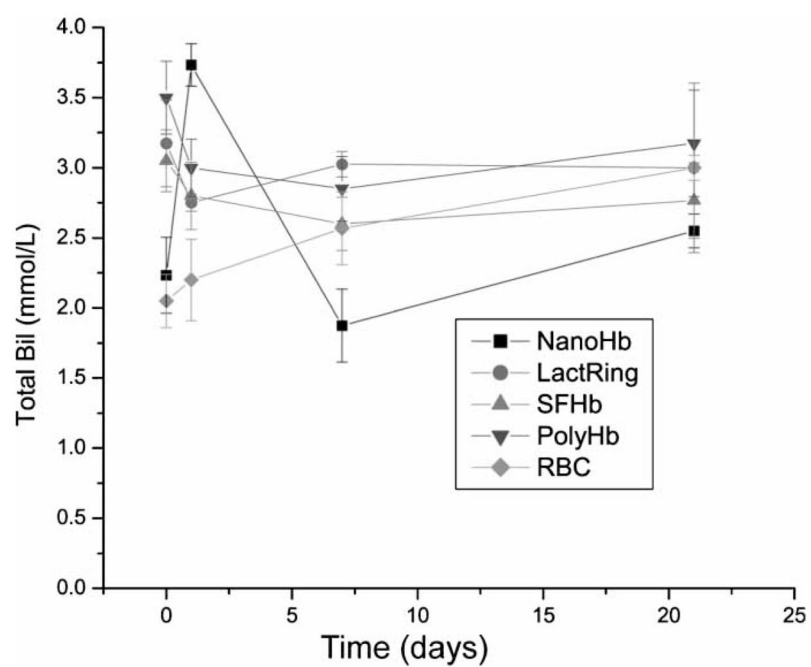


Figure 3. 33% toploading of Nano artificial RBCs, LactRing, SFHb, PolyHb, or RBC does not result in elevation of the systemic levels of total bilirubin in rats. (Mean \pm S.E.).

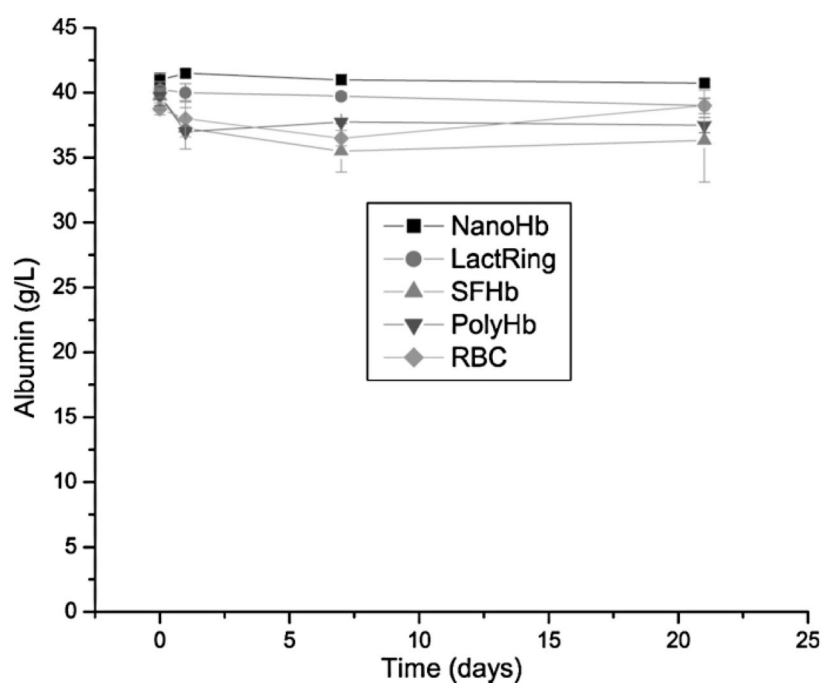


Figure 4. The albumin level for the Nano artificial RBCs group remained the highest of all the solutions infused. (Mean \pm S.E.).

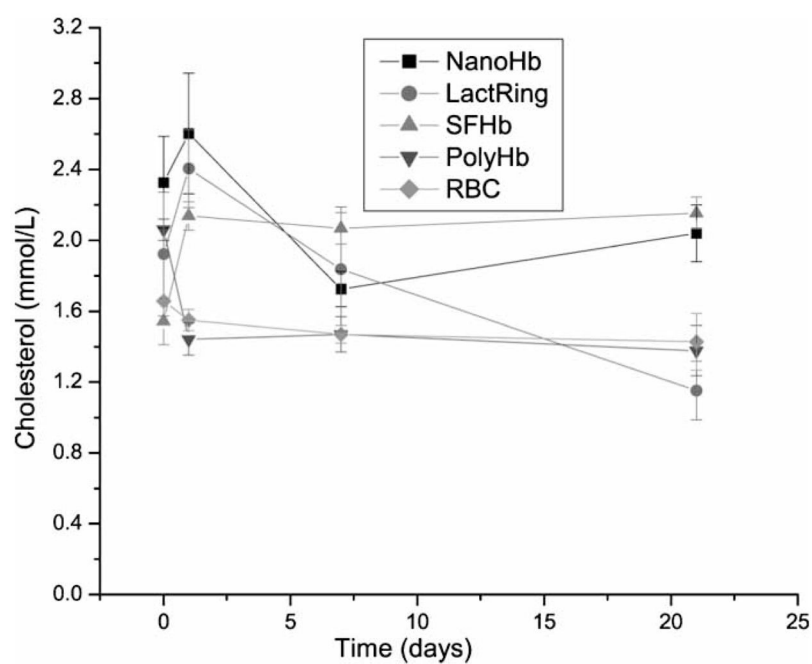


Figure 5.

There was no elevation of the systemic cholesterol level for the groups receiving 33% volume topholding of Nano artificial RBCs, LactRing, SFHb, PolyHb, or RBCs. (Mean \pm S.E.).

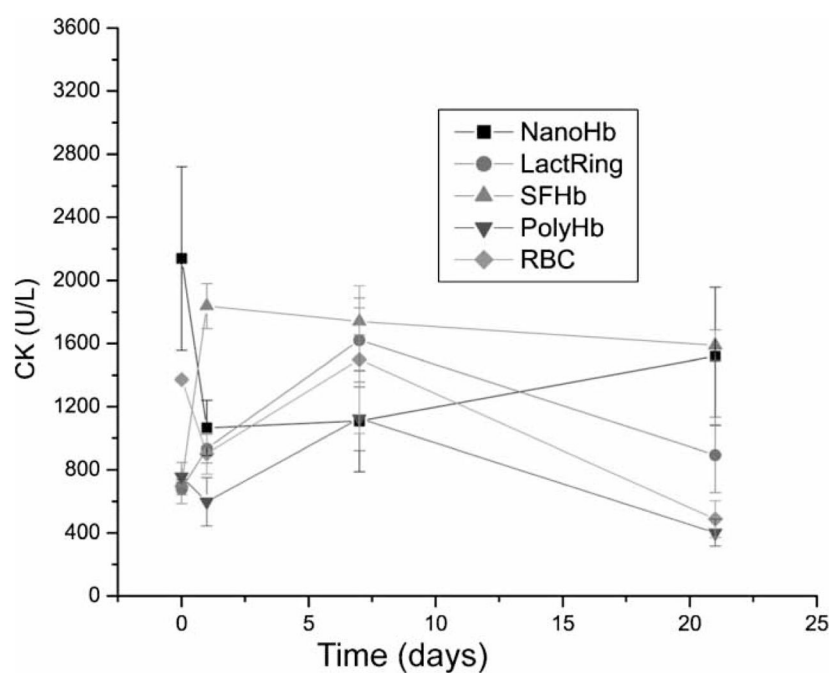


Figure 6. Creatine kinase levels in rats infused with 33% volume of Nano artificial RBCs, LactRing, SFHb, PolyHb, and RBCs. (Mean \pm S.E.).

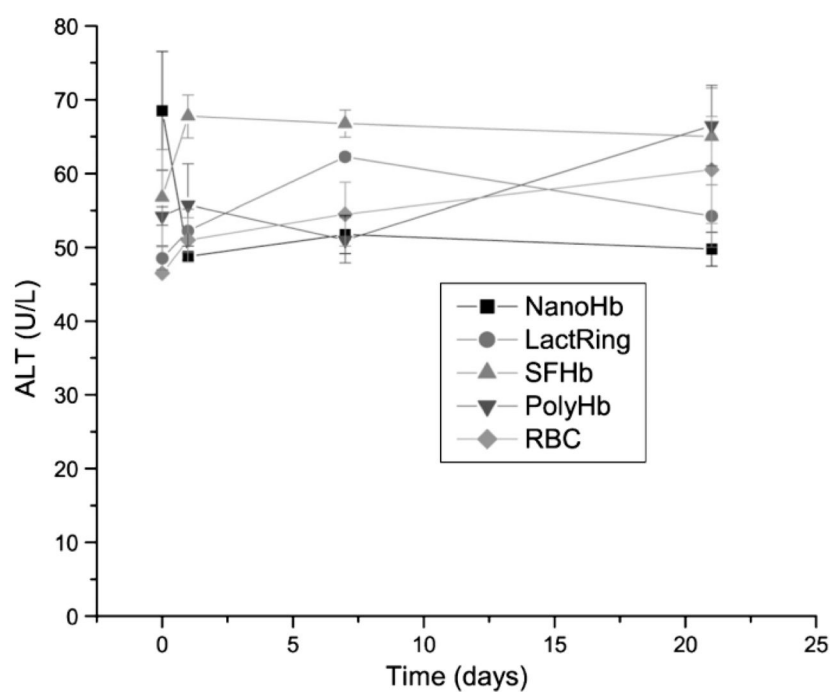


Figure 7. ALT levels in rats infused with 33% volume of Nano artificial RBCs, LactRing, SFHb, PolyHb, and RBCs. (Mean \pm S.E.).

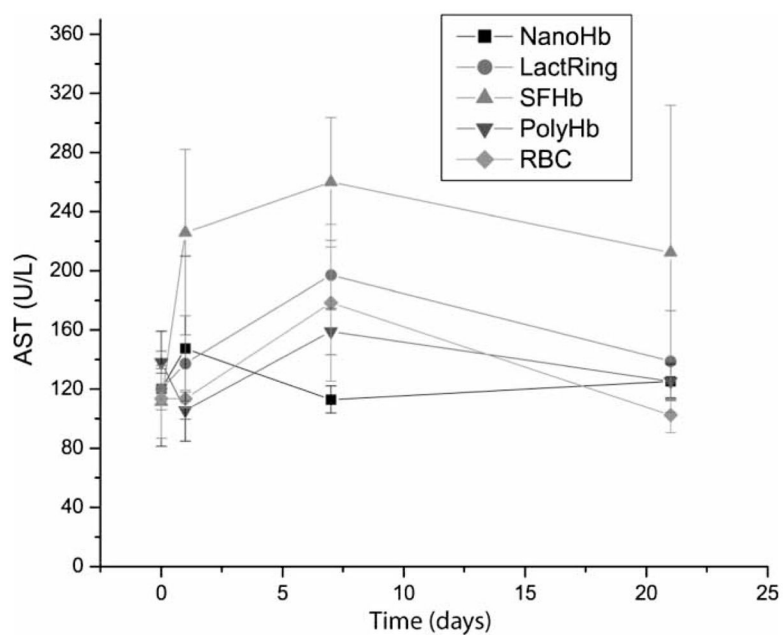


Figure 8. AST levels in rats infused with 33% volume of Nano artificial RBCs, LactRing, SFHb, PolyHb, and RBCs. (Mean \pm S.E.).

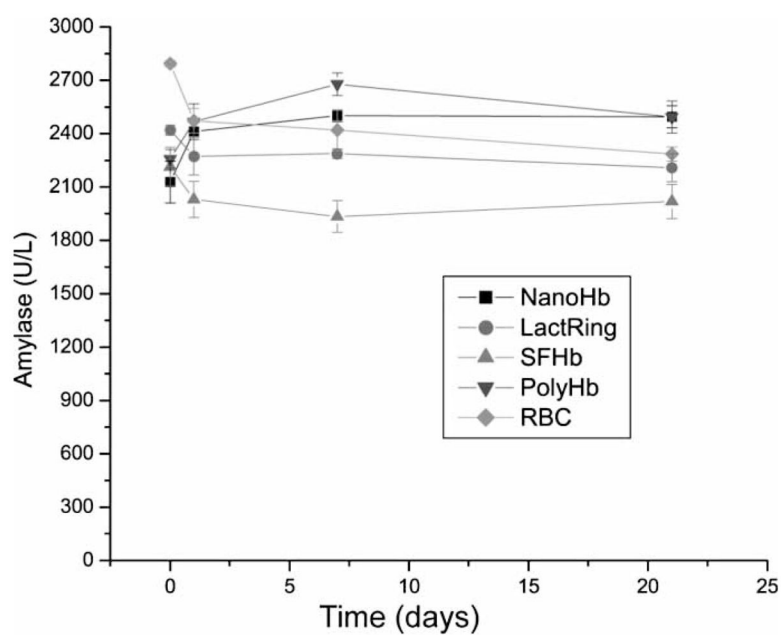


Figure 9. Creatine kinase levels in rats infused with 33% volume of Nano artificial RBCs, LactRing, SFHb, PolyHb, and RBCs. (Mean \pm S.E.).

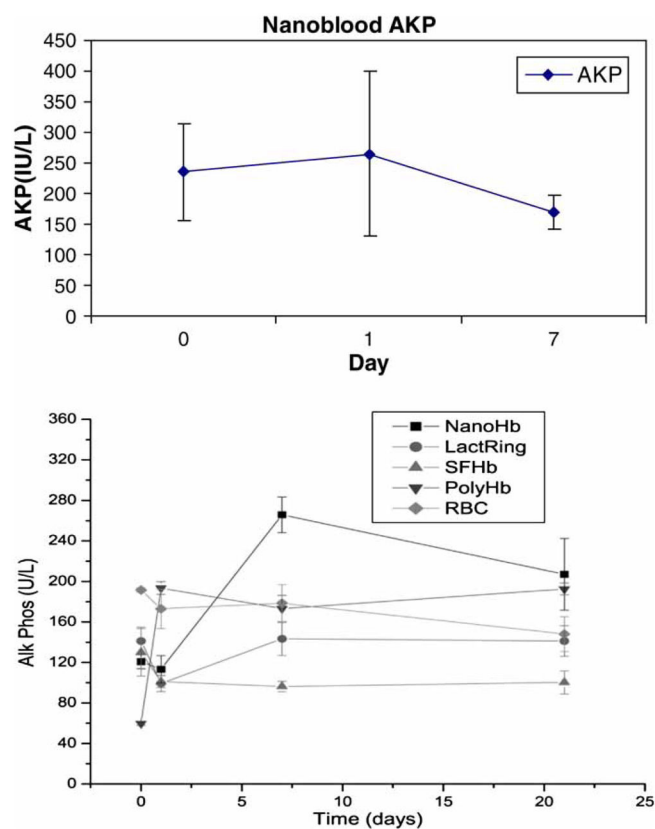


Figure 10.

Alkaline Phosphatase (AKP) levels in rats infused with 33% volume of Nano artificial RBCs, LactRing, SFHb, PolyHb, and RBCs. (Mean \pm S.E.).