In vivo toxicity screening assay of Nanomotors

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ABSTRACT

In vivo toxicity screening is a vital part of the clinical trial for any therapeutics. In vivo studies are generally conducted on lower mammals like rats or mice. The assay performed here involves the toxicity screening of helical magnetic nanomotors in BALB/c mice model. This study focuses on quantifying the toxic effect of these nanomotors and to determine the LD_{50} dose using Up & Down method of mathematical analysis and different serum analysis tests such as SGOT, SGPT and Bilirubin quantification to analyze the damage to liver or other tissues in the experimental model.

Keywords: Nanomotors, toxicity, LD_{50}

1. INTRODUCTION

Pre-clinical trials are a vital process the clinical trials of any therapeutics. The determination of LD_{50} value is a very important part of pre-clinical trials. LD_{50} is the dose concentration of a substance at which fifty percent of the test population dies. The determination of this value is important for the determination of safe dosage for the drug or any other compound and to reduce side effects.

The test material is 2 μm long helical magnetic nanomotors made up of SiO_2, Fe, Ag, Au and Ti. This study aims to find toxicity of these nanomotors and finding any damage to organ functionality, particularly liver. The assays chosen to determine these effects are SGOT, SGPT and Bilirubin level testing. An increased level of these biochemical parameters in the blood of test mice essentially indicate damage or reduction of organ functionality.

The determination of LD50 dosage involves a statistical approach known as the Up & Down method. This method aims at reducing the number of test organisms and also helps to predict a fairly accurate lethal dose using various mathematical functions [1].

In nacroparticles, toxicity shows significant correlation to the mass of particles injected. But in micro and nano scale toxicity depends on a multitude of factors as seen from the literature review such as

type of particle, size and shape, specific surface area, charge, coating, dispersion, agglomeration, aggregation, concentration, number of particles and mode of administration. Existing literature report toxicity studies in micro-nanoparticles of size range 10 nm to 10 um, made of gold (Au), silver (Ag) and silica (SiO_2). But in this study, the nanomotors are composite particles containing iron and titanium in addition to all of these materials. Since the properties of these nanomotors vary considerably with respect to their constituents, the results available on the constituent materials cannot be extrapolated and considered to be relevant to the present study. Further, most of the literature analyses spherical particles while the particles in this study are helical structures with aspect ratio of 10:1. This necessitates the need of a separate in vivo toxicity study for these nanomotors.

OECD 425 guidelines were followed throughout the experimental procedure and the parameters of evaluation, dosage formulation, variables such as temperature, humidity, feed, bedding etc. were maintained according to these guidelines.

2. PROCEDURES AND PRINCIPLES

2.1 Biochemical toxicity analysis:

2.1.1 SGOT/AST – For the quantitative determination of Aspartate aminotransferase in serum

Aspartate aminotransferase measurements are used in the diagnosis and treatment of certain types of liver and heart disease. The AST reagent is used to measure aspartate aminotransferase activity by an enzymatic rate method. In the assay reaction, the AST catalyzes the reversible transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase (MDH) with the concurrent oxidation of β-Nicotinamide Adenine Dinucleotide (reduced form) (NADH) to β-Nicotinamide Adenine Dinucleotide (NAD)[2]. The system automatically proportions the appropriate sample and reagent volumes into a cuvette. The ratio used is on part sample to 11 parts of reagent. The system monitors the rate of change in absorbance at 340 nm over a fixed-time interval. This rate of change in absorbance is directly proportional to the activity of AST in the sample and is used by the system to calculate and express the AST activity.

2.1.2 SGPT/ALT - For the quantitative determination of Alanine Aminotransferase in serum.

ALT is widely distributed in tissues with the highest concentrations found in the liver and kidneys. Even so, ALT is considered more liver-specific than AST. Elevated levels of ALT are often only observed in liver diseases such as cirrhosis, hepatitis, or metastatic carcinoma [3]. However, there can be elevated levels of ALT with infectious mononucleosis, muscular dystrophy, and dermatomyositis. ALT catalyzes the transfer of the amino group from L-alanine to α-ketoglutarate resulting in the formation of pyruvate and L-glutamate. Lactate dehydrogenase catalyzes the reduction of pyruvate and the simultaneous oxidation of NADH to NAD. The resulting rate of decrease in absorbance is directly proportional to ALT activity.

ALT

L-Alanine + α-Ketoglutarate ---------- Pyruvate + L-Glutamate

LDH

Pyruvate + NADH + H+ ----------L-Lactate + NAD+ +H2O

2.1.3 Bilirubin

Bilirubin, a degradation product of heme catabolism, is a non-polar molecule. There are two forms of bilirubin: water-soluble (conjugated or direct) and water-insoluble (unconjugated or indirect) bilirubin. Bilirubin is produced in the endoplasmic reticulum as unconjugated bilirubin, which binds to albumin in plasma and forms albumin-bilirubin complex. This complex is transported to the liver, where it is conjugated with glucuronic acid and forms conjugated bilirubin. Bilirubin has potent antioxidant, anti- inflammatory and autoimmune properties. Bilirubin concentration in human body depends on gender, drug intake, age, etc. Low serum bilirubin is directly correlated with pathological conditions including diabetes mellitus, metabolic syndrome, and cardiovascular diseases [4]. However, high bilirubin indicates hemolysis, jaundice, Gilbert's syndrome, hepatitis, drug toxicity, and possible blockage of bile ducts.

2.2 Up and Down method

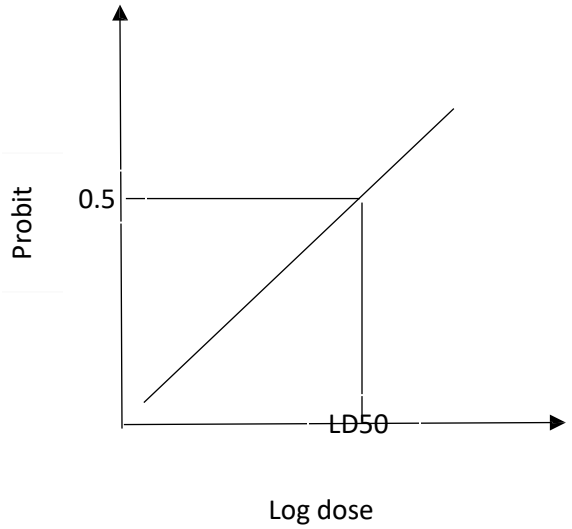
The traditional methods used for LD_{50} estimation are Litchfield and Wilcoxon method, Molinengo method, Thompson and Weil method or, alternatively, the maximal non-lethal dose and the approximate lethal dose[5]. However all of the aforementioned methods use a considerably large number of animals for the studies and also a wide range of drug concentrations are used. The Up and Down method on the other hand starts with a certain calculated dose and then either goes higher or lower until the LD_{50} is determined. This system leads to the use of a relatively lower number of test animals and also a limited number of concentrations of drugs. Thus it is widely used in determination of LD_{50} values for different medicines, chemicals and other substances that are introduced in a living system for a therapeutic or diagnostic purpose.

The up & down method follows a statistical approach which reduces the number of test animals that are required to perform the toxicity analysis. The first animal to be tested is dosed a step lower than the estimated LD_{50} dose. Post the dose injection, the animal is observed for 48 hours. Standard parameters like activity, skin and fur, diarrhea, eyes, body weight, mucous membrane, salivation, sleep or coma, tremors are observed regularly and noted. If the animal is alive after 48 hours and does not show any abnormalities in any of the aforementioned parameters, the dose that is administered to the next animal is increased. Contrastingly, if the animal dies within the observation period, the dose to the next animal is reduced. The factor by which the dosage is increased or decreased is 3.2. The dose progression factor should be chosen to be the antilog of 1/ (the estimated slope of the dose-response curve) and should remain constant throughout testing (a progression of 3.2 corresponds to a slope of 2). When there is no information on the slope of the substance to be tested, a dose progression factor of 3.2 is used [6].

Dosing continues depending on the fixed -time interval (e.g., 48-hour) outcomes of all the animals up to that time. The testing stops when one of the following stopping criteria first is met:

(a) 3 consecutive animals survive at the upper bound;
(b) 5 reversals occur in any 6 consecutive animals tested;
(c) At least 4 animals have followed the first reversal and the specified likelihood-ratios exceed the critical value. Calculations are made at each dosing, following the fourth animal after the first reversal.

After the data is obtained, a probit vs log dose graph is plotted and this linear graph gives us the accurate LD50 value for the substance being tested.



2.3 Hematoxylin and Eosin staining

As the nature of the study aims at finding the adverse effects besides the LD50 of the nanomotors, it is important to analyze the various tissue samples such as Liver, Spleen and Lung tissue. These are the primary regions where the nanomotors are estimated to be deposited once they have been injected into an in-vivo model, thus it is vital to perform a histopathological analysis of these tissues. Hematoxylin and Eosin staining method is suitable for the analysis of the effect of the nanomotors on the tissue.

Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex reaction. Eosin is pink in colour and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Well-fixed cells show considerable intra-nuclear detail. If abundant polyribosomes are present, the cytoplasm will have a distinct blue cast. The Golgi zone can be tentatively identified by the absence of staining in a region next to the nucleus. Thus, the stain discloses abundant structural information, with specific functional implications. A limitation of hematoxylin staining is that it is incompatible with immunofluorescence. It is useful, however, to stain one serial paraffin section from a tissue in which immunofluorescence will be performed [7].
To perform a comparative assay, we obtain tissue samples from both dead mice and mice that were alive after being administered the nanomotors and then proceed to section the tissue and stain the same using H&E staining protocol. These stained tissue samples when compared to tissue samples of perfectly healthy control mice would give us an idea about the nature and extent of changes caused due to the nanomotors and any tissue damage, if present can be identified easily.

3. METHODOLOGY

Healthy female BALB/c (20-29g) were purchased from CAF (Central Animal Facility), IISc Bangalore. They were separated in plastic cages with stainless steel mesh lids in a ventilated room. The room was maintained at around 25 ℃ and 45% to 60% relative humidity with a 12 h light-dark cycle. The animals were kept in their cages for at least 5 days prior to dosing, to allow for their acclimatization to the laboratory conditions

Preparation of nanomotors:

The wafers with standing nanomotors were sonicated in PBS for 30s and were injected into the mice at concentrations of 2.5×10^8 (17.5mg/kg), 6.25×10^8 (55 mg/kg) nanomotors per mouse. The mice received 0.4 ml Nanomotors i.e 100μL of nanomotors by intravenous injection via the tail vein at half an hour interval. The control group received 100μL PBS instead of nanomotors. The mice were monitored continuously for first 4 hours post injection and periodically observed for 24 hours, 48 hours, at least once daily for any changes in their behavior for a period of one week. Blood samples were collected by retro orbital method using micro-capillary tube. The serum was obtained by centrifugation of the whole blood at 8,000 rpm for 10 min. Liver function was evaluated based on the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and Total Bilirubin test.

4. RESULTS

4.1 Up & down method:

Dose 1 (17.5 mg/kg) –

Parameters	Control 1	Control 2	Test 1	Test 2
Skin and fur	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Hypo-activity	Nil	Nil	Nil	Nil
Tremors	Nil	Nil	Nil	Nil
Diarrhea	Nil	Nil	Nil	Nil
Death	Nil	Nil	Nil	Nil

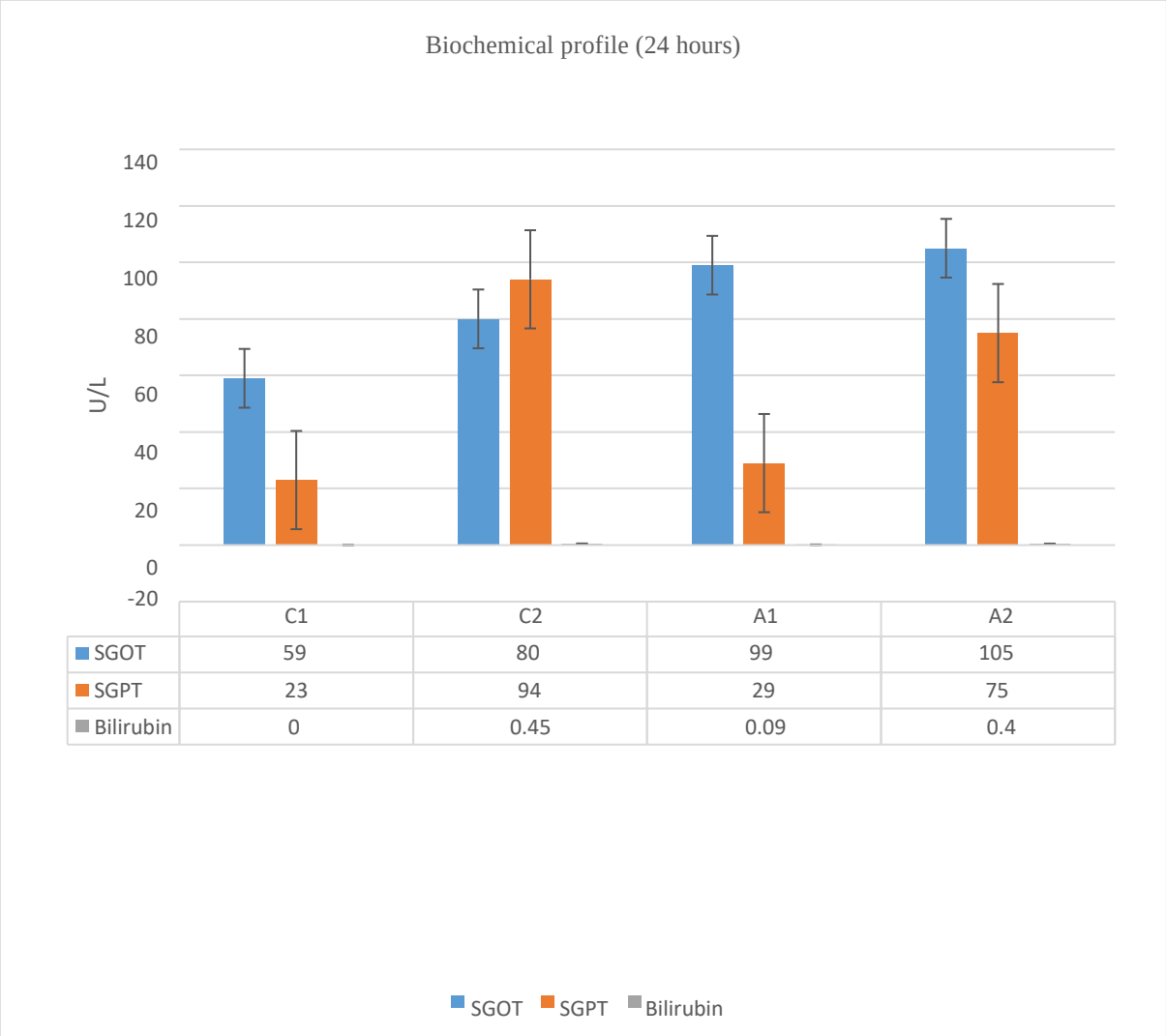
Table 1: Table representing different parameters for control and test mice for one week after injecting.

Dose 2 (55 mg/kg)-

Parameters	Control 1	Control 2	Test 1	Test 2
Skin and fur	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Hypo-activity	Nil	Nil	Nil	Nil
Tremors	Nil	Nil	Nil	Nil
Diarrhea	Nil	Nil	Nil	Nil
Death	Nil	Nil	Nil	Nil

Table 2: Table representing parameters observed for second batch of test mice one week after second dose injection.

4.2 Biochemical analysis:



	C1	C2	A1	A2
SGOT	59	80	99	105
SGPT	23	94	29	75
Bilirubin	0	0.45	0.09	0.4

Figure 1: Figure representing values of SGOT, SGPT and bilirubin 24 hours after injecting with a dose of 17.5 mg/kg of body mass (2.5 x 10^8 Nanomotors). Values are represented as mean ± SEM.

Normal range: SGOT: (54 – 298) SGPT: (17-77) Bilirubin (0-0.9 mg/ml)

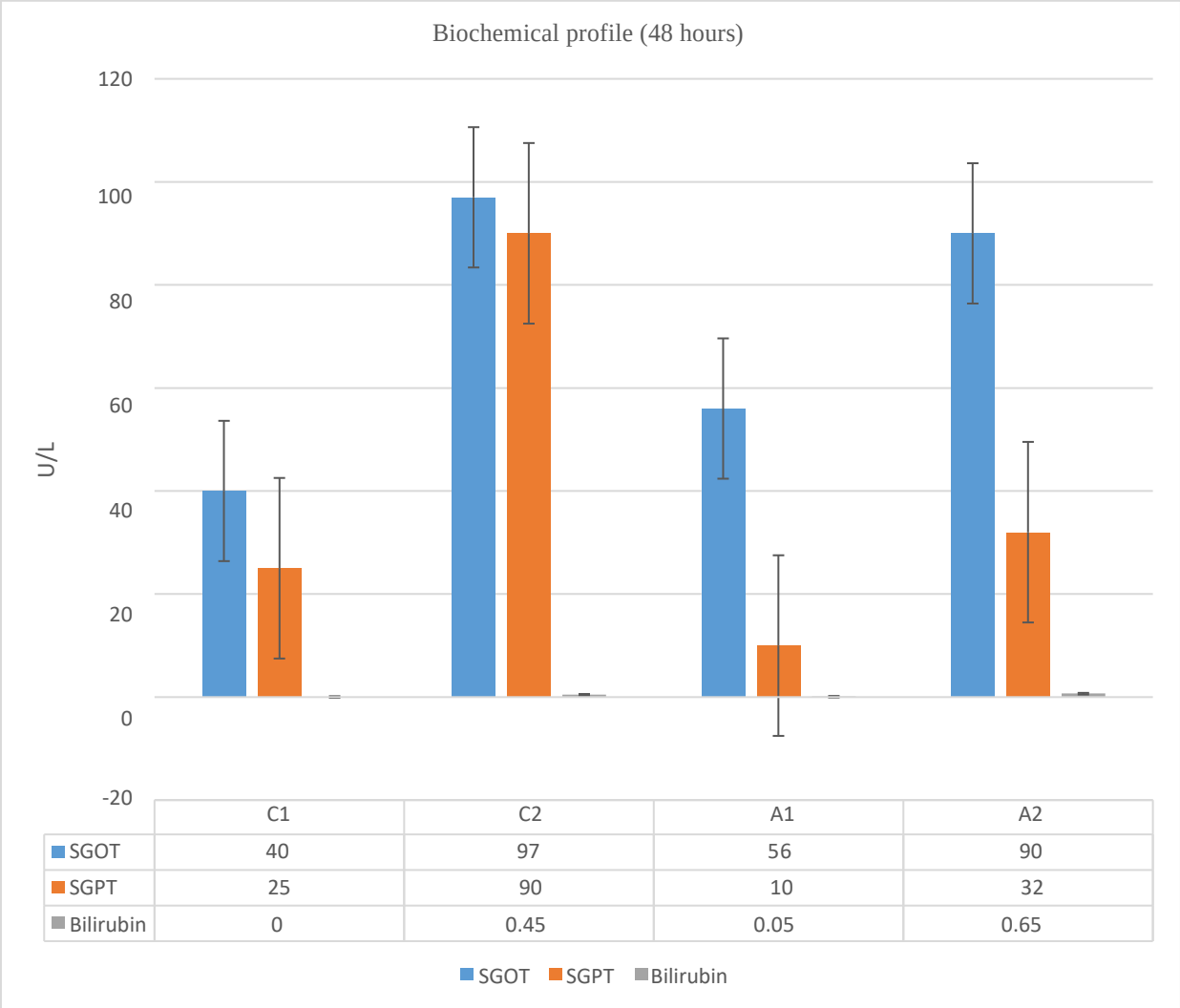


Figure 2: Figure representing values of SGOT, SGPT and Bilirubin 48 hours after injecting with a dose of 17.5 mg/kg of body mass (2.5×10^8 Nanomotors). Values are represented as mean ± SEM.

Normal range: SGOT: (54 – 298) SGPT: (17-77) Bilirubin: (0-0.9 mg/ml)

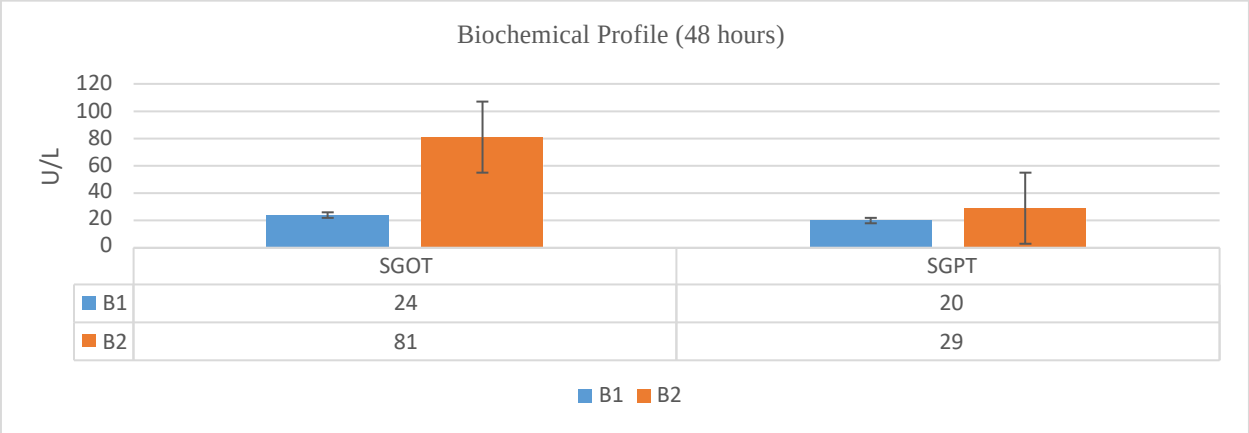


Figure 3: Figure representing values of SGOT and SGPT 48 hours after injecting with a dose of 55 mg/kg of body mass (6.25 x 10^8 Nanomotors). Values are represented as mean ± SEM.

Normal range: SGOT: (54 – 298) SGPT: (17-77) Bilirubin: (0-0.9 mg/ml)

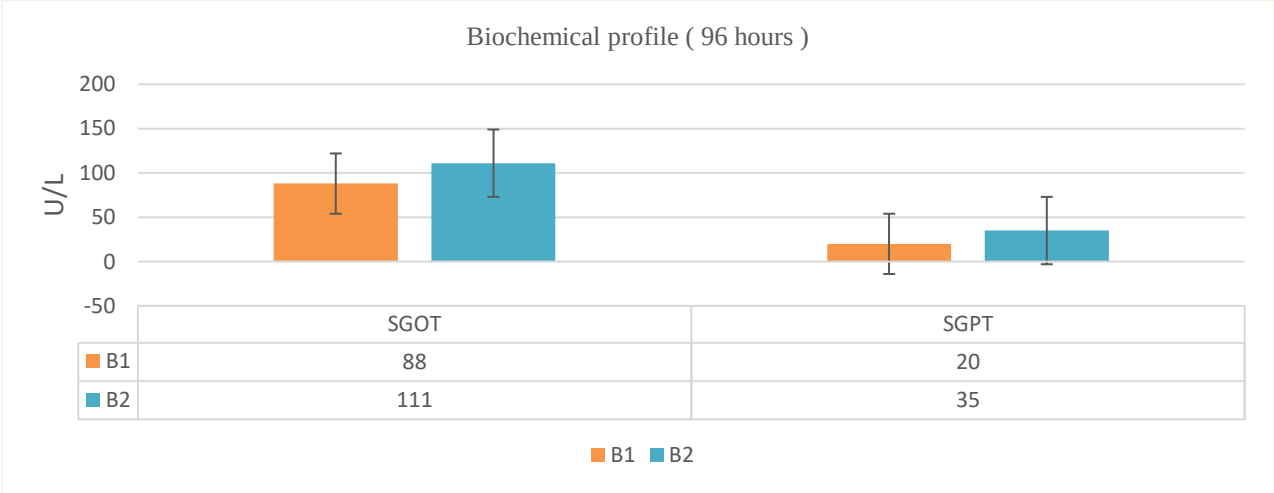


Figure 4: Figure representing values of SGOT and SGPT 96 hours after injecting with a dose of 55 mg/kg of body mass (6.25 x 10^8 Nanomotors). Values are represented as mean ± SEM.

Normal range: SGOT: (54 – 298) SGPT: (17-77) Bilirubin: (0-0.9 mg/ml)

5. CONCLUSION

Based on the result from blood biochemistry assay, different doses of nanomotors i.e 17.5 mg/kg and 55 mg/kg were found to be non-toxic and the mice did not show any changes in its behavioral pattern upto 48 hrs.

The biochemical profiling also shows that the AST (54-298 U/L), ALT (17-77 U/L) and Bilirubin (0-0.9 U/L) values in the treated mice are within normal range. This suggests that there is no damage in the hepatic, muscle and cardiac tissue upto 48 hrs.

Depending on the dosages injected in the two sets of test animals, we can conclude that the nanomotors do not confer any toxic effects in BALB/c mice in the range of 10^8 nanomotors upto 48 hrs. Further increase in dosage is required to determine the LD_{50} value. ICP- MS (Inductively coupled plasma-mass spectrometry) and histological examination of the organs will be subsequently conducted in order to identify the cause of death and bio-distribution of the nanomotors.

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7. REFERENCE

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