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Safety Evaluation Study of Repaglinide Loaded Polymeric Nanoparticles Formulation.

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ABSTRACT

Nanotechnology has facilitated noteworthy advances in the field of medicine. Whereas a number of nanoparticle drug combinations are at various stages of preclinical or clinical assessment, the careful assessment of their toxic response is the need of the hour. This study aims to evaluate the safety profile of Poly (lactic-co-glycolide) (PLGA) nanoparticles containing the antidiabetic drug, Repaglinide (Rg) for its effective oral delivery. The Rg loaded polymeric nanoparticles were synthesized and characterized for particle size, drug content, entrapment efficiency and compatibility. The nanoparticles were then fed to wistar albino rats for a duration of 21 days, to assess the adversities of nanoparticles on various organs in the rats. Along with the biochemical and haematological studies, histopathological examination was done to monitor intravascular effects, and they were also tested for haemocompatibility and cytotoxicity. MTT measurement reveals that the nanoparticle treated cells are 70-80% viable. The test drug did not produce any significant histopathological changes in the cryoarchitecture of any organ studied at various dose levels. Hence our data indicates that these polymeric nanoparticles are nontoxic and provide a suitable platform for the oral delivery of the antidiabetic agent.

Keywords: Repaglinide, nanoparticles, histopathology, cytotoxicity, antidiabetic.

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INTRODUCTION

Nanotoxicology is emerging as an important sub discipline of nanotechnology and the field is budding without any ending spectacle [1]. It's not possible to say nanoparticles are safe, and it's not possible to say nanoparticles are dangerous, it lies somewhere in the middle and it depends on the specifics [2]. Nanotoxicology refers to the study of harmful interactions of nanostructures with biological systems with an emphasis on elucidating the relationship between the physical and chemical properties of nanostructures with induction of toxic biological responses. An understanding of relationship between the physical and chemical properties of the nanostructure and their *in-vivo* behavior would provide a basis for assessing toxic response [3]. The surface area to mass is increased as the size of any particle decreases. The toxicity of any particle is related to its surface area because chemical reactions occur on the surface of materials, not within them. Therefore, nanoparticles that enter the bloodstream, if toxic, have the ability to cause significantly higher levels of toxicity than larger molecules would be expected to. Due to these unique properties, it is impossible to look at the Material Safety Data Sheet (MSDS) of a substance and extrapolate those risk characteristics to the nanoscale level because the identical substance at the nanoscale level has unique properties, and therefore substantially unique risks, different from the original substance. Effects from ingestion of nanoparticles were not well understood. There is currently little to no data regarding the possible effects from administration of nanoparticles. Health effects of nanoparticles are attracting considerable and increasing concern of the public and government worldwide. Uptake of particles of different size via the gastrointestinal tract can also lead to different toxicological effects [4, 5]. But the reports about toxicological research of nanomaterials by the gastrointestinal tract are few [6]. Nanoparticles, products of nanotechnology, are of increasing interest to the pharmaceutical community. Despite many advances in the development of oral hypoglycemic agents, an ideal drug for treating diabetes mellitus is still a distant reality. Controlled release formulations should be developed taking into consideration the patient's habits and their physiological responses. Extended release formulations with improved dissolution properties, and particularly extended release formulations of antidiabetic drugs, are therefore desirable additions to the medical treatment of diabetes, including type II diabetes [7]. Recently many studies are focused on safety issues of manufactured nanomaterials to minimize or eliminate their nanotoxicity before they are being widely used [8, 9].

Toxicity studies to assess the safety of this compound for human consumption have become a prerequisite for the promotion of the product. Till date, no such polymeric nanoparticle formulation toxicity study of repaglinide with PLGA has been reported for oral use. The oral route of drug administration is generally preferred because of its versatility, safety and relative patient comfort. Hence, there is an outstanding need of research for polymeric nanoparticles to find whether they are stable for prolonged shelf life. The present study concentrates mainly on the toxicity assessment of formulated polymeric nanoparticles. A rigorous safety of these nanoparticles would enable their use in the field of diabetic therapy.

MATERIALS AND METHOD

Materials

Repaglinide was obtained from Micro Labs ltd, Hosur, Poly (lactide-co-glycolide) [PLGA] with an average molecular weight of 20,000 with copolymer ratio of lactide to glycolide of 75:25, has been procured from Sigma Aldrich, Germany. Dichloromethane was supplied by Ranbaxy Fine chemicals Ltd, New Delhi, India. The other chemicals used were of analytical grade.

METHODOLOGY

Preparation of polymeric nanoparticles

Polymeric nanoparticles were prepared by solvent evaporation method using PLGA as polymer coating material and Repaglinide as the core material. Weighed quantity of drug and polymer were dissolved in suitable organic solvent i.e., Dichloromethane (organic phase). This solution was added drop by drop to the aqueous phase of PVA and homogenized using IKA T 25 Digital Ultra turrax homogenizer, at 24000 rpm followed by magnetic stirring for 3 hours. The formed nanoparticles were recovered by centrifugation (Sigma centrifuge) at 25,000 rpm for 15 minutes followed by washing thrice and lyophilized.

Characterization of polymeric nanoparticles

The shape and surface morphology of the nanoparticles were examined using Scanning Electron Microscopy (SEM) (JSM - T20. Tokyo, Japan). Particle size was determined using Photon Correlation Spectroscopy (PCS) (Malvern S4700 PCS System, Malvern UK). Drug Content (% w/w) and Drug Entrapment (%) were determined by using formula [10].

Haemolytic Assay

The test samples were made by preparing stock solution of nanoparticle formulation using phosphate buffer as the solvent followed by incubation. Various concentrations of the formulation i.e., 20, 30, 40, 50µg in 0.5ml were used for the study [3]. Haemolytic assay was carried out by adopting the method of Bulmus [11].

Cytotoxicity Assay

Normal kidney cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 100units/mL penicillin, 100µg/mL streptomycin and 0.25µg/mL ampotericin B (antibiotic-antimycotic kit; GIBCO In vitrogen, Carlsbad, CA, USA) and incubated at 37°C under 5% CO_2. For the toxicity assay, three day cultured cells were seeded into a 96 well plate (Griener, Solingen, Germany) at a density of $6x10^4$ cells/well. Cells in the wells were incubated for 24hours with varying final concentration of nanoparticle formulations. Some of the untreated wells served as control. Cells were incubated with 10µL of 5mg/ml MTT (Sigma, St. Louis, MO, USA) solution for 2hours at 37°C. Cell lysis buffer (20%wt/vol SDS in a solution of 50% dimethylformamide, pH 7.4) was added, and the plate was incubated overnight at 37°C. Optical densities (OD) at 570nm were measured using extraction buffer as a blank. Because MTT is metabolized, via the mitochondria, to formazan by living, viable cells, a reduction of measured OD at 570nm compared to untreated healthy control cells indicates a loss of cell growth and viability. The percentage viability was determined by dividing the optical density of the treated cells by that of the control cells and multiplying by 100. Each experiment was performed three times.

Sub Acute Oral Toxicity Study in Wistar Albino Rats

All procedures using animals were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) and the protocol of animal study was IAEC 03/003/08. Sub acute oral toxicity was conducted with one rodent species (rat). Wistar female albino rats (120-125g) used for this study were procured from King Institute, Guindy, Chennai, India and housed in the Institutional animal house under standard environmental conditions (23±1˚C, 55±5% humidity and 12h/12h light/dark cycle) and maintained with free access to standard diet (Hindustan Lever Ltd, Bangalore, India) and water *ad libitum*. Females were nulliparous and non pregnant. Then animals were housed in poly propylene cages with stainless steel grills, sieved and sterilized paddy husk used as bedding. Bedding material, cages, grills and water bottles were changed on alternate days. Three rats with same sex were housed in a group wise manner. After acclimation, animals were randomized into control (n=6) and two experimental groups (n=12, each group n=6). In this study, wistar albino rats were administered with polymeric nanoparticles following Organization for Economic Cooperation and Development (OECD) test guideline 425, based on a repeated oral dose toxicity study applying Good Laboratory Practices. The food and water were analyzed and are considered not to contain any contaminants that could reasonably be expected to affect the purpose or integrity of the study.

Study Design

The study design of sub acute oral toxicity is given in Table 1. The animals were divided into 3 groups (n=6) with a total of 18 animals and the following regimen of treatment was followed. Sub acute toxicity was estimated by mortality and survival time, as well as by clinical picture of intoxication including behavioral reactions. Animals on study were observed for any adverse reaction, such as change in body weight, stool, condition of eye and nose, motor activity, as well as neuromuscular reactions etc. All animals examined for internal abnormalities viz. size, weight and appearance of brain, heart, lungs, liver, spleen and kidneys were assessed at necropsy [12]. Rats were bled via the retro orbital plexus and the blood was collected in prelabelled EDTA and heparinised vials for haematological and biochemical analysis respectively, before sacrificing.

Table 1: Study design of sub acute oral toxicity experiment

Group	Description	Dose mg/kg body weight	Number of animals (Female)	Sacrifice timings
1	Control	saline	6	21 days
5	Rg- PLGA(1:3)	5	6	21 days
6	Rg–PLGA(1:3)	10	6	21 days

Changes in Body Weight, Feed and Water Consumption

The mean body weight of all the animals was taken from the start of the study to the final sacrifice day. The quantity of food consumed by groups, consisting of 6 rats each, was recorded daily, and food consumption was calculated for control and treatment groups [13].

Post Observation of the Animals

Observations of pharmacotoxic signs were made at 10, 30, 60 and 120 minutes and at 4 and 6 hours after dosing, during the first day and daily thereafter for 21 days. The time onset, intensity, and duration of symptoms, if any, were recorded. All animals were observed twice daily for mortality during the 21 days period of study. Appearance of skin, fur, eyes, respiratory, autonomic and CNS were observed during these days. At the end of 21st day all the animals were sacrificed humanely and observed for gross pathology.

Gross pathology and changes in internal organs weight

Gross pathological examination was carried out and the following organs were examined at necropsy- lungs, heart, liver, spleen, kidneys, brain and pancreas.

Haematological analysis

Red blood cells and white blood cells content of the blood samples were determined in a haemocytometer. Haematocrit (HCT) and haemoglobin (Hb) contents were estimated spectrophotometrically using standard methods. Platelet count was made using direct method and clotting time by capillary method. For differential count, blood smear was stained with leishman stain and leucocytes were counted under light microscope. All other haematological parameters such as mean corpuscular volume (MCV), mean corpuscular/cell haemoglobin concentration (MCHC), lymphocytes and neutrophils were estimated in the haematology analyzer (BC 2800Vet Mindray, Germany).

Biochemical assays

The blood samples were centrifuged at 2000 rpm for 5 minutes using sigma centrifuge. The serum was kept at -80°C until analyzed. Levels of serum glutamate oxaloacetic transaminase (SGOT), serum alkaline phosphatase (SAP), serum glutamic pyruvic transaminase (SGPT), serum creatinine, serum bilirubin, proteins and minerals were determined with an automatic analytical instrument (Hitachi 911, Japan) [3].

Histopathology

Internal organs of the experimental animals were fixed in 10 % formalin, embedded in paraffin and cut into 5µm thick sections in a microtome. Sections were mounted on glass slides using standard techniques. After staining with hematoxylin – eosin, the sections were examined and photographed under a light microscope equipped for photography (Olympus CK 40).

Statistical Analyzes

The statistical package used was SPSS 15.0 software. Student t test was used for comparison between control and experimental groups. Values were expressed as mean±S.E.M and those values with $P<0.05$ were considered significant. For graphs Origin software 6.0 version was used.

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RESULTS

Formulation and Characterisation of nanoparticle:

The polymeric nanoparticles were prepared and the formulation having drug: polymer ratio of 1:4, showing nanoparticle recovery of 91.62%, was considered to be optimum for further studies. It showed a drug content of 18.70 % with an encapsulation efficiency of 80.2%. The size of the nanoparticles ranged within 384 nm. The characterization results for all the formulations are tabulated in Table 2.

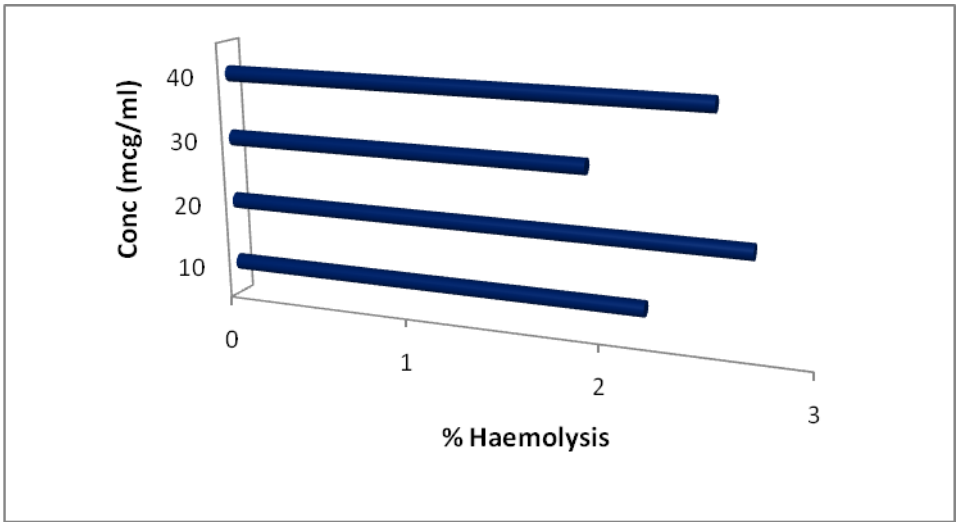
Table 2: Characterization report of Repaglinide polymeric nanoparticle formulations

Drug Polymer ratio	Nanoparticle recovery (%)	DC (%w/w)	EE (%)	Size Distribution (nm)	Polydispersity index	Zeta potential
1:2	91.3	8.43	77.0	555±50	0.921±0.09	-2±20
1:3	91.44	8.57	78.40	785±75	1.000±0.12	+2±5
1:4	91.62	18.70	80.2	384±20	0.956±0.08	-4±10

Haemolytic Assay

The haemolytic assay of formulation in different ratios with different concentration was performed and the results are shown in Figure 1. Haemolytic activity did not show any observational toxicity. The data obtained in this assay gives a qualitative indication of the damage caused by polymeric nanoparticles in red blood cell. Obviously, the result declared that the nanoparticles were more haemocompatible for drug delivery applications.

Figure 1: Haemolytic assay of RP-PLGA polymeric nanoparticle



Cytotoxicity Assay

Figure 2 shows the results of MTT assay of polymeric nanoparticle formulations. Cytotoxicity assay by MTT measurement reveals that the nanoparticle treated cells are 70-80% viable for the whole duration of the assay period. Cell cytotoxicity was studied at concentration ranging from A- 10µg/ml, B- 20µg/ml, C-30µg/ml, and D-40µg/ml for the formulations. The result implies that the nanoparticle formulations are non toxic even at higher doses.

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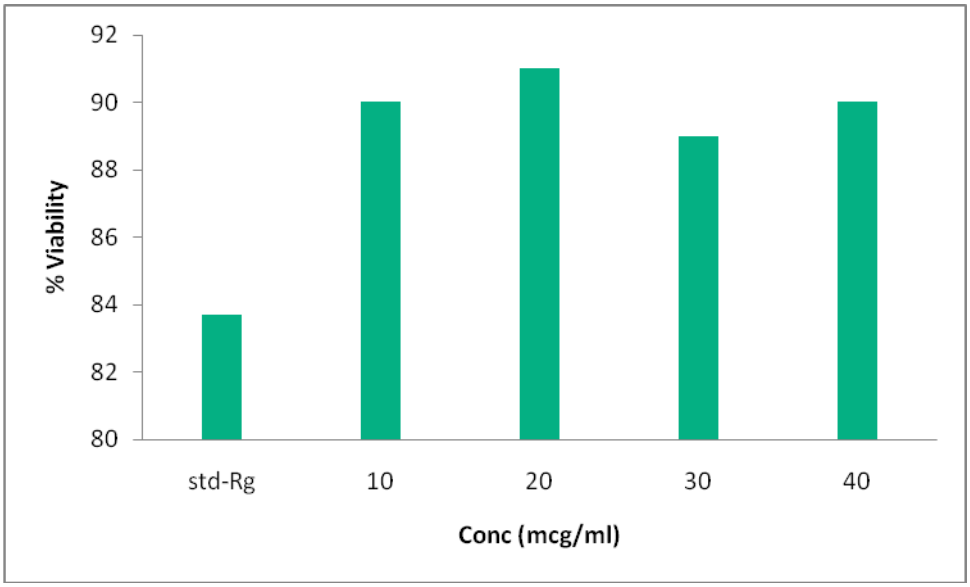
Figure 2: Influence of nanoparticle formulation on the cytotoxicity of normal kidney cell lines



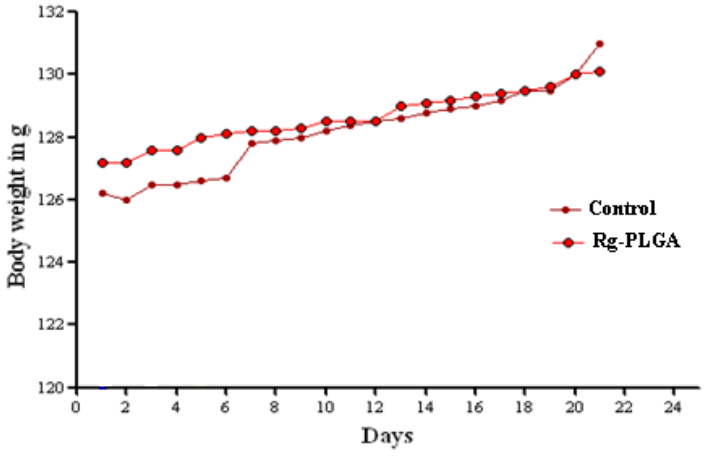
***In-vivo* Toxicity Studies- Animal Observation**

All animals survived until study termination. Cage side observations revealed no signs of toxicity. Oral administration of drug loaded polymeric nanoparticles did not cause abnormality during the experimental period. It is noteworthy that significant behavior changes like salivation, piloerection, clonic and tonic movement abnormality, edema, nostril discharge etc. were not observed in any of the test groups and these parameters remained unaffected throughout the entire study.

Changes in Body Weight, Feed and Water Consumption

This study revealed that there were no significant changes in body weight gain between the control and experimental group. The purpose of the current chapter is to look at the toxicity profile of the formulated nanoparticles. 30 and 21 day study is considered as a sub chronic study, which is well accepted for eliciting any toxicity on long term feeding. These nanoparticles at various concentrations and ratios did not appear to retard growth or affect food consumption and utilization. Animals remained healthy throughout the study period; the average weight gain at the end of the study in control and nanoparticle treated group are shown in the Figure 3.

Figure 3: Changes in the body weight gain of rats administered with Repaglinide polymeric nanoparticles for 21days compared with control group. Control, Rg-PLGA(5mg/kg bw).



Values shown are mean (n=6).

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Gross Pathology and Changes in Internal Organs Weight

Gross examination of the internal organs of all rats revealed no detectable abnormalities. Plus, no significant organ weight changes were observed between treatment and control groups after 21 days of administration (Table 3). A marginal but statistically non significant decrease in weight of liver was observed in test drug administered groups in comparison to control groups. Thus, it can be concluded that nanoparticle formulation is virtually nontoxic.

Table 3: Changes in the internal organ weight of Rg polymeric nanoparticle treated animals compared with control

Organs ¥	Control	PLGA Nanoparticles	
		5 mg	10 mg
Lungs	1. 5 ± 0.1	1.26±0.1	1.4±0.1
Liver	6.87±.15	5.89±1	6.4±.86
Kidney	0.56±.3	0.89±0.12	0.78±.3
Spleen	0.54±0.3	0.59±0.7	0.6±0.1
Brain	1.85±0.2	1.9±0.2	1.6±0.3
Heart	0.7±0.5	0.7±0.5	0.8±0.3
Pancreas	0.3±0.25	0.5±0.2	0.3±0.5

All values are expressed in mean ± SEM (n=6). No significance compared to control

Haematological Analysis

The status of bone marrow activity and intravascular effects were monitored by haematological examination as summarized in Table 4. Nevertheless, all values lay within normal limits for this animal species [14].

Table 4: Haematological report of Repaglinide polymeric nanoparticle treated rats compared with control rats after 21 days daily oral administration

Parameters	Control	Rg-PLGA	
		5 mg	10 mg
Haemoglobin (g%)	15.2±0.1	14.1±1	14.2±1
WBC (cells/cm)	9083±30	6900±126	7500±450
Polymorph (%)	55±1.8	67±1	56±3
Lymphocytes (%)	31.8±1.1	31±1.6	32±3
Eoisnophils (%)	2.2± 0.4	2.6±0.8	2.2±0.1
Monocytes (%)	1±0.01	1.2±0.1	1±0.9

All values are expressed in mean ± SEM (n=6). No significance compared to control

Biochemical assays

The nanoparticles treated group did not show any damage to the liver, kidney and pancreas as examined by clinical blood chemistry. The results are summarized in Table 5. Nonetheless, these values are within the normal range [15]. The present study did not find any statistical difference in enzyme activity between the control and the experimental group. Mean values of SAP, SGOT and SGPT between control and experiment groups was not statistically significant but, an increased level of enzyme values was observed in all groups.

Table 5: Biochemical report of Repaglinide polymeric nanoparticle treated rats compared with control rats after 21 days daily oral administration

Parameters	Control	Rg -PLGA	
		5 mg†	10 mg†
Serum creatinine (µmol/L)	0.9 ±0.1	0.98±0.1	0.97±0.76
Total bilirubin (µmol/L)	0.9±0.12	0.9±0.3	0.8±0.2
SAP(IU/L)	129 ±2.17	134±1.6	132±2
Proteins(g/L)	7.5±0.17	7.2±0.5	6.2±0.9
SGPT(IU/L)	27±0.8	29±3	31±2
SGOT(IU/L)	32.6±1	32±2.2	34±2
Glucose(mg/dl)	103±23	99±12	98±10
Cholesterol(mg/dl)	85±2	70±4	83±5
Triglycerides(mg/dl)	68±4.2	67±4.3	69±2.1
Urea(mg/dl)	17±2	17±2.9	19±2
Sodium(mmol/L)	141±1	134±4	145±3
Chloride(mmol/L)	107±2.5	112±2.4	109±3.2
Potassium(mmol/L)	4.25±0.08	4.2±0.56	3.9±0.78
Bicarbonates(mmol/L)	26.5±0.56	34±0.89	29±0.6

† - Dose mg/kg body weight. All values are expressed in mean ± SEM (n=6)

Histopathological Examinations

The test drug did not produce any significant changes in the cryoarchitecture of any organ studied at various dose levels. There were no apparent gross lesions at necropsy and histological examination of vital organs did not reveal any pathological changes. In liver, no sinusoidal distension but mild vacuolar degeneration of hepatocytes was observed but that findings are not much apparent. Kidney showed mild interstitial nephritis and tubular cell degeneration in some animals but not in all groups. The lungs, spleen and brain were normal. Pancreatic islets are distinguished. The lobular architecture is maintained with variable admixture of acini, islets and ductal structures in pancreas. Since, the biochemical parameters showed good result indicating normal hepatocellular and nephrotic function, the pathological changes were not of serious nature. Hence, it may be concluded that the test drug has no serious toxicity producing potential in most of the organs studied. Figure 4 shows the histopathological slides of rats treated with Rg-PLGA nanoparticles.

DISCUSSION

Nanoparticles prepared for drug delivery always need special attention. There is no universal "nanoparticle" to fit all the problems, each nanoparticle prepared by using different polymers should be treated individually when health risks are expected. In fact, related toxicity issues are often ignored. Irrespective of the uptake route, the body distribution of particles is most dependent on the surface characteristics and the size of the particles. It is an important issue in drug design in order to help deliver the medication to the right target. In unintentional uptake of nanoparticles, these characteristics can strongly influence the accumulation of a specific type of particle in the particular body site. Our research is focused on the medical applications of nanotechnology including the toxicity associated with their use. Despite the widespread use of nanoparticles for drug delivery, understanding of the toxicity and potential health risks associated with these nanoparticles use is extremely limited. Some authors reported the toxicity related to nanoparticles [16]. Thus, along with the development of novel nanoparticles, experts in related scientific fields are calling for a simultaneous assessment of the toxicological effects of nanoparticles [17].

Colloidal drug carrier systems serve to minimize the side effect of drugs [18, 19]. Side effects often result from the destruction of corpuscles of blood or tissue at the site of action and these may be reduced by incorporating the drug in colloidal carriers. Many haemolytic assays are employed for formulation testing *in-vitro* [20]. Haemolytic assays were performed because formulation possessing potent activity in the form of nanoparticle may not be useful in pharmacological aspects if they possess haemolytic effect. The extent of haemolysis is an important parameter of toxicity of formulated nanoparticles to erythrocytes [10, 21].

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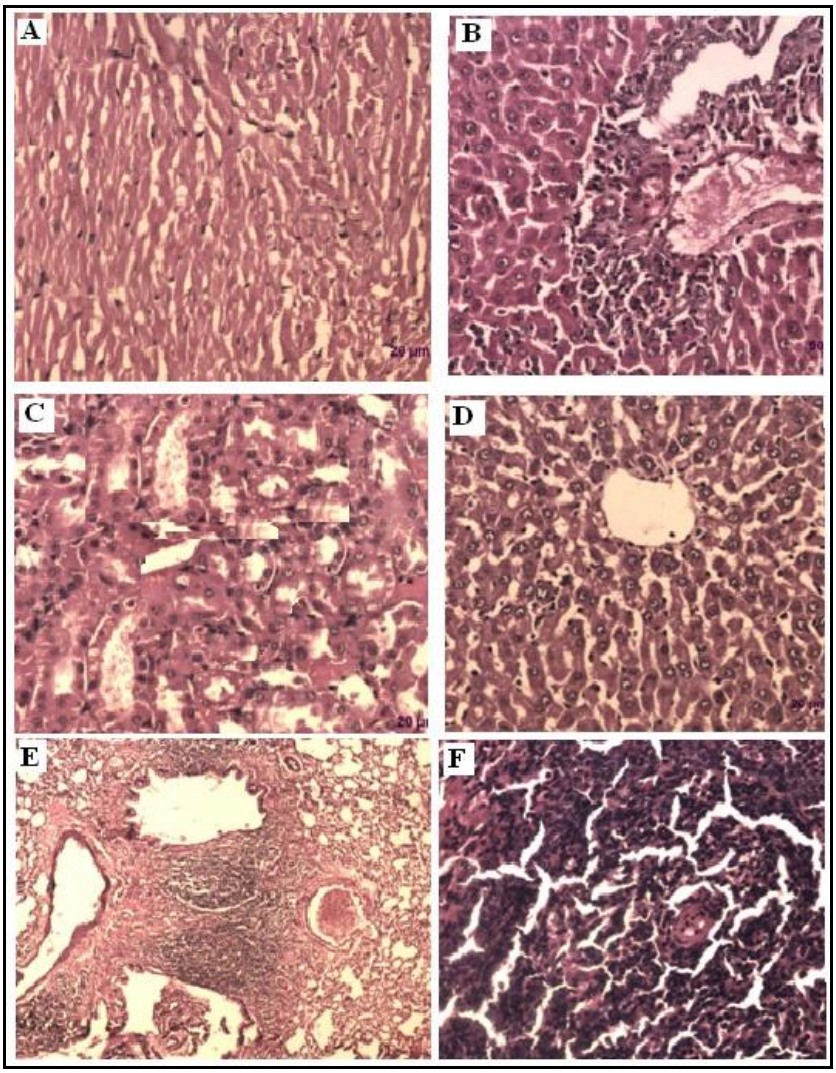
Figure 4: The histopathological slides (magnification ×100) of Wistar albino rats treated with nanoparticle formulation. Slides, A-Heart, B-Kidney, C-Liver, D-Liver, E-Lungs, F-Spleen histosection of rat administered with 5mg/kg body weight of Rg-PLGA polymeric nanoparticle.



The successful development of *in-vitro* assays as predictive screens for assessing particle toxicity is an important goal during early product development. If properly validated, the advantages of the early screening tests like haemotoxicity would be useful. Very few systematic attempts have been made to validate the results from *in-vitro* studies to *in-vivo* toxicity effects on the same materials. Then what is the necessary to go for an *in-vivo* study is always a question. The reason is in fact most of the drug particles and other agents that were determined to be in the least toxic range following assessment using *in-vitro* assay methodology were ironically, the most toxic following administration into the animals [22]. Many studies concluded that *in-vitro* assays may provide cell type specific mechanistic information, but do not accurately reflect *in-vivo* comparative toxicity results [23].

The data obtained in haemolytic assay gives a qualitative indication of the damage caused by polymeric nanoparticles in red blood cell. The result obviously declared that the nanoparticles are more haemocompatible for drug delivery applications. The nanoparticles were administered into the body for drug delivery or oral detoxification, detrimental interaction of these particles with blood constituents must be avoided. In the present study, the nanoparticle dispersion does not show any observational haemolytic toxicity in the red blood cell and haemolytic percentage of the nanoparticle dispersions was independent of its concentration. All the formulations showed haemolytic percentage which was significantly lower than 5%. These results suggested that polymeric nanoparticles were suitable for a wide safety margin in blood contacting applications and oral administration. Cytotoxicity assays are widely used by the pharmaceutical

industry. All formulations and new drug, screened for unwanted cytotoxic effects before investing in their development as a pharmaceutical. It helps to remove potentially toxic compounds early in the drug discovery process or formulation. The present study result implies that the nanoparticle formulations are non toxic even at higher doses.

Normal body weight gain was observed in the control as well as the test drug administered groups. However, none of the changes were statistically significant. Our findings indicate that these polymeric nanoparticles are nontoxic during the study period and therefore have potential for safe use in oral formulations. Toxicity was constantly observed after the oral administration of nanoparticles to rats [13]. No modification in animal behavior was observed and at the post mortem examination no clinical signs or organ abnormality was detected. The feed consumption of the different groups followed a similar pattern indicating the normal metabolism of the animals. Changes in body weight gain are an index of toxicity after exposure to toxic substances [24, 25]. The administration of polymeric nanoparticles did not create any significant hematological changes. MCV, MCH, MCHC, HCT, Clotting time and ESR values also remains normal in all the experimental animals. Feeding of these nanoparticles did not alter the levels of glucose, protein, bilirubin, or creatinine indicating normal hepatocellular and nephrotic function. Thus, the present study observations do not suggest that the nanoparticle formulation produces toxicity in sub acute period. The evaluation of drug carriers also has to focus on the toxicity of such systems [13]. PLGA is a slow biodegradable polymer made up of L-lacticide, a nontoxic, natural by- product of anaerobic metabolism of glucose [26]. Therefore, adequate experimental data regarding toxicity is necessary for all the nanodrug delivery system. Our study demonstrated that oral administration of PLGA nanoparticles loaded with repaglinide had fine biological compatibility without any toxic effects in the rats. No histopathologic changes were observed in treated groups compared with normal group as a control. From histopathological analysis, it could be confirmed that drug loaded PLGA polymeric nanoparticle did not seriously damage the organ by accumulation within organ. These findings indicated the safety of the oral gavage method and the polymer materials.

CONCLUSION

In the present investigation, the toxicity of nanoparticle has been studied in order to demonstrate the safety profile. There were no treatment related mortalities, clinical signs of toxicity throughout the course of the study. The test drug has no serious toxicity producing potential and no observed adverse effect level (NOAEL). Therefore, it can be concluded that PLGA nanoparticles loaded with drug could be available as drug carrier system for the treatment of diabetes. Until now, studies on the adverse outcome of nanoparticles were limited to the experimental stage and further detailed studies are required for more complete understanding.

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