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Original Article

Preliminary Toxicological Report of Metformin Hydrochloride Loaded Polymeric Nanoparticles

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ABSTRACT

Nanosized materials have tremendous application in every field of human activity, with a lot of economic benefit increasing nanoparticle research and use. There are number of nanosized products already available commercially and many others are in queue. Therefore, there is a pressing need for careful consideration of benefits and side effects of the use of nanoparticles in medicine. This research work aims at providing a balanced update of this exciting potentially toxicological effect of manufactured Metformin hydrochloride loaded polymeric nanoparticles. To assess the toxicities systematically on the functions of various tissues and organs in rats, the rats were fed with the manufactured polymeric nanoparticles for a period of 30 days repeated oral administration. Variation in the protein, carbohydrate and fat metabolic profile of the rat exposed to nanoparticles were studied by hematobiochemical and pathology profiles. The haemolytic potential of these nanoparticles were determined by means of an *in vitro* haemolysis assay. All formulations showed haemolytic effect less than 5%. The study revealed that Metformin loaded PMMA and PLGA polymeric nanoparticle did not produce any toxicity.

Key words: Haemolysis, metformin hydrochloride, polymeric nanoparticles, toxicity

INTRODUCTION

The nanotechnological field is growing without any end in sight. Nanoparticle drug delivery systems are nanometeric carriers used to deliver drugs or bio molecules.[1] "It is a mistake for someone to say nanoparticles is safe, and it is a mistake to say nanoparticles are dangerous. They are probably going to be somewhere in the middle, and it will depend very much on the specifics."[2] Nanotoxicology is emerging as an important sub discipline of nanotechnology. Nanotoxicology refers to the study of the interactions of nanostructures with biological systems with an emphasis on elucidating the relationship between the physical and chemical properties (e.g. size, shape, surface chemistry, composition, and aggregation) of nanostructures with induction of toxic biological responses. An understanding of the relationship between the physical and chemical properties of the nanostructure and their *in vivo* behavior would provide a basis for assessing toxic response. In this article, we provide a rationale for *in vivo* animal studies to assess nanotoxicity. It is important to understand the fate of the drugs once delivered to the nucleus and other sensitive cells organelles. Furthermore, because nanosystems increase efficiency of drug delivery, the doses may need recalibration.[3] Nevertheless, the future remains exciting and wide open.

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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

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bloodstream, if toxic, have the ability to cause significantly higher levels of toxicity than larger molecules would be expected to. 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.[6,7]

Metformin hydrochloride is a drug used for treating type 2 diabetes mellitus. Metformin improves hyperglycemia primarily through its suppression of hepatic glucose production (hepatic gluconeogenesis).[8] Toxicological effects of prepared nanoparticles for medical applications are poorly understood, despite carefully controlled studies by respected scientists and institutions. In order to expand our knowledge of polymeric nanoparticle loaded with drug and to assess further systematically, we performed the study to observe the toxic effects of nanoparticle on the functions of various tissues and organs in rats. In case of Poly (lactide-co-glycolide) (PLGA) and Poly (methyl methacrylate) (PMMA) polymeric nanoparticle formulations we were using organic solvents and the permitted amount by FDA is less than 10 ppm and hence toxicity study for these nanoparticle formulation is a compulsion. In this article we are explaining the behavioral, cytotoxic, hematobiochemical and pathology profiles of metformin loaded polymeric nanoparticles after repeated oral administration for 30 days to wistar female albino rats.

MATERIALS AND METHODS

Drugs and chemicals

Metformin hydrochloride 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, Poly (methyl methacrylate) (PMMA) and Poly (Vinyl Alcohol) (PVA) have been procured from Sigma Aldrich, Germany. Dichloromethane, Acetone and Petroleum ether were supplied by Ranbaxy Fine chemicals Ltd, New Delhi, India. The other chemicals used were of analytical grade and are manufactured in India.

Preparation of polymeric nanoparticles

Polymeric nanoparticles were prepared by solvent evaporation method using different polymers separately as coating material and Metformin as core material. Weighed quantity of drug and polymer were dissolved in suitable organic solvent i.e., Dichloromethane for PMMA and PLGA polymer (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 hrs. The formed nanoparticles were recovered by centrifugation (Sigma centrifuge) at 25,000 rpm for 15 minutes followed by washing thrice and lyophilized.[9]

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.[9]

Haemolytic assay

Measuring haemolytic activity is important as it is an indicator of cytotoxicities. The *in vitro* haemolysis test has also been employed by many different groups for their toxicological evaluation. It gives a quantitative measure of the haemoglobin release.[10,11] The test samples were made by preparing stock solution of nanoparticle formulation using phosphate buffer as solvent followed by incubation. Various concentration of the formulation i.e., 20,30,40,50 μg in 0.5 ml were used for the study. Haemolytic assay was carried out by adopting the method of Bulmus.[10] Freshly collected rat red blood cells (RBC) were taken and washed three times by 150 mM Sodium Chloride (NaCl) (2500 rpm for 5 minutes). After removing NaCl at the last wash step the cells were suspended in 100 mM Sodium phosphate buffer. The test samples were mixed with 200 μL of RBC solutions and the final reaction mixture volume was made up to 1 ml by adding Sodium phosphate buffer. The reaction mixture was then placed in water bath for 1 hr at 37°C. After the incubation time the reaction mixture was centrifuged again at 2500 rpm for 10 minutes. Measure the supernatant absorbance at 541 nm keeping sodium phosphate buffer as blank. Deionised water was used as a positive control. The experiment was done in triplicate and percentage haemolysis was calculated using the following formula.

$$\text{Percentage haemolysis} = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of positive control}} \times 100$$

Animals

Wistar female albino rats (120-150 gms) 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 12 hrs/ 12 hrs light/dark cycle) and maintained with free access to standard diet (Hindustan Lever Ltd, Bangalore, India) and water *ad libitum*. Then animals were divided into seven groups, each group containing 6 animals (n = 6 per groups) and housed in poly propylene cages. The protocol of animal study was approved by Institutional Animal Ethics Committee (IAEC 03/003/08). In this study,

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Wistar albino rats were exposed to polymeric nanoparticles following Organization for Economic Cooperation and Development (OECD) test guideline 425, based on a 30- day repeated oral dose toxicity study applying Good Laboratory Practice.

Toxicity studies

The sub acute toxicity of drug loaded nanoparticles was framed out. The animals were divided into three groups ($n = 6$) and following regimen of treatment was followed.

Group 1- Treated as control, received normal saline per oral daily for 30 days.

Group 2- Treated as test, received dose of 5 mg/kg body weight of Metformin hydrochloride- PLGA formulation per oral daily for 30 days.

Group 3- Treated as test, received dose of 5 mg/kg body weight of Metformin hydrochloride- PMMA formulation per oral daily for 30 days.

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.[12,13] Rats were bled via the retro orbital plexus before sacrificing. 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 dose groups.[9]

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).[14]

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 gluatmic pyruvic transaminase (SGPT), serum creatinine, serum bilirubin, proteins and minerals were determined with an automatic analytical instrument (Hitachi 911, Japan).[9]

Histopathology

Internal organs of the experimental animals were fixed in 10% formalin, embedded in paraffin and cut into 5 μm 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

Statistical analysis

The data were subjected to statistical analysis by applying one way analysis of variance (ANOVA) using Statistical package for social sciences (SPSS), 13 version.

RESULTS AND 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, toxicity issues related 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. 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.[15,16] 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]

Metformin has an oral bioavailability of 50–60% under fasting conditions, and is absorbed slowly. Peak plasma concentrations (C_{max}) are reached within one to three hours of taking immediate-release metformin and four to eight hours with extended-release formulations. Metformin nanoparticles were prepared by solvent evaporation method. The average size of the nanoparticles was smaller than 300 nm and the size distribution exhibited a narrow and monodisperse pattern. The amount of drug introduced and the ratio of loading between polymer and drug were investigated according to our previous papers. The results are more reliable (data not shown). In this study, we mainly focused on toxicity of drug loaded nanoparticles prepared using PMMA and PLGA polymer as drug carriers since

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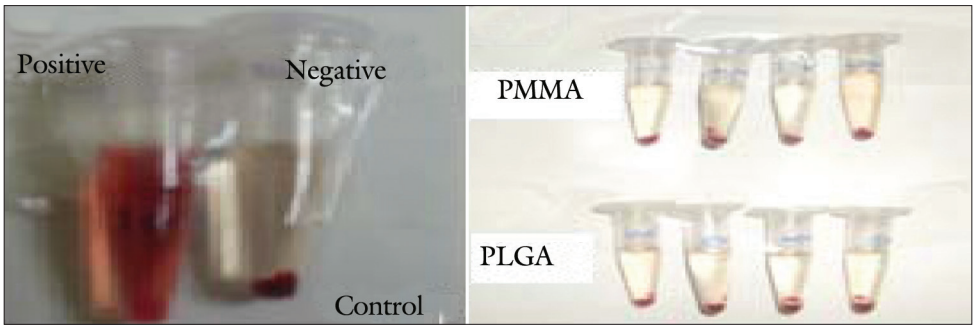


Figure 1: Observation of A. Positive (haemolysis) and negative (non-haemolysis) of control, B. Non- haemolytic nature of formulated PMMA and PLGA metformin nanoparticle

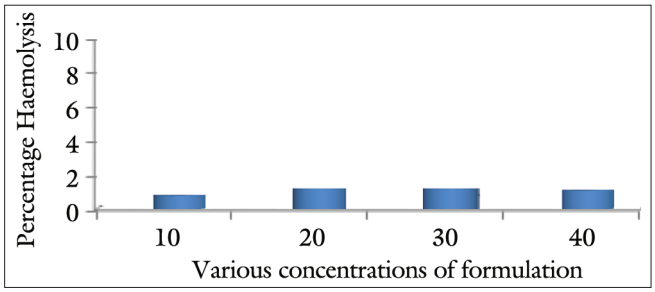


Figure 2: Percentage haemolysis of various concentrations of Metformin PMMA polymeric nanoparticle in rat red blood cell

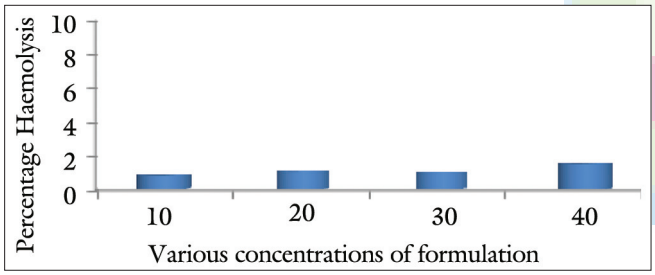


Figure 3: Percentage haemolysis of various concentrations of Metformin PLGA polymeric nanoparticle in rat red blood cell

such polyesters were well tolerated and also known to be biodegradable but the biodegradability of PMMA is still a question. The data obtained in haemolytic assay gives a qualitative indication of the damage caused by polymeric nanoparticles in red blood cell [Figures 1-3]. The result obviously declared that the nanoparticles are more haemocompatible for drug delivery applications. Moreover, the nanoparticle system showed lysis less than 5% in the whole experimental concentration range. The extent of haemolysis is an important parameter of toxicity of formulated nanoparticles to erythrocytes. Polymeric nanoparticles with different types of polymer have different functional groups. The charge is crucial for toxicity including hemotoxicity. It has been documented that mammalian cell (RBC) toxicity arises from the formation of conducting pores in the cell membrane thus changing membrane permeability or it can be due to the alteration of sodium potassium and calcium magnesium ATPase activities.[18]

Table 1: Effect of polymeric nanoparticle in Wistar albino rats

Parameter	Results
Motor activity	Normal
Clonic and Tonic movements	Normal
Pilo Erection	Negative
Righting Reflex	Positive
Lacrimation	Normal
Salvation	Normal
Respiration	Normal
Skin colour	Normal
Muscle spasm	Negative
Touch response	Normal
Urination and defecation	Normal

Table 2: Biochemical report of polymeric nanoparticle treated animals after 30 days daily oral administration compared with control animals

Parameters	Control	Metformin PMMA	Nanoparticles PLGA
Creatinine (mg/dl)	0.6 ± 0.05	0.59 ± 0.1	0.71 ± 0.1
Total Bilirubin (mg/dl)	1.5 ± 0.3	1.54 ± 0.5	1.52 ± 3
SAP (U/l)	458.3 ± 33	395 ± 12	398 ± 20
Proteins (g/dl)	6.9 ± 0.2	7.8 ± 0.5	7.3 ± 0.6
SGPT (U/l)	87.3 ± 2.33	77.5 ± 2.3	72.5 ± 0.6
SGOT (U/l)	203.7 ± 6.2	205 ± 2	179 ± 2
Glucose (mg/dl)	92.8 ± 10.7	98 ± 5	101 ± 3
Cholesterol (mg/dl)	71.3 ± 18.4	60 ± 14	65 ± 2
Triglycerides (mg/dl)	76.7 ± 11.5	89 ± 3	98 ± 5
Urea (mg/dl)	39.2 ± 2.2	35 ± 2	34.5 ± 2
Sodium (mmol/L)	140.3 ± 1.4	145.6 ± 2	135 ± 3
Chloride (mmol/L)	103.7 ± 3.7	110.5 ± 2	115 ± 2
Pottassium (mmol/L)	3.5 ± 0.1	3.9 ± 1	3.5 ± 1.2
Bicarbonates (mmol/L)	26.5 ±0.6	25 ± 2.3	23 ± 2

All values are expressed in mean ± SEM (n = 6)

Toxicity was constantly observed after the oral administration of nanoparticles to rats. No modification in animal behavior was observed and at the post mortem examination no clinical signs or organ abnormality was detected [Table 1-2]. There was no significant effect on body weight and feed

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Table 3: Haematological report polymeric nanoparticle treated animals after 30 days daily oral administration compared with control animals

Parameters	Control	Metformin Nanoparticles	
		PMMA	PLGA
Heamoglobin (gms/dl)	12.2 ± 0.27	13.6 ± 0.3	13 ± 0.1
WBC (Cells/cubic mm)	11.6 ± 0.36	17 ± 0.2	12 ± 0.9
Polymorph %	7.40 ± 2.2	6.5 ± 7	7 ± 0.7
Lymphocytes %	87.93 ± 0.32	72.6 ± 9	71 ± 0.8
Eosinophil %	2.2 ± 0.33	1.7 ± 1	2 ± 0.1
Monocytes %	2.4 ± 0.45	2.5 ±1	2 ± 0.1
RBC (millions/cubic mm)	7.95 ± 0.43	8.4 ± 0.1	8 ± 0.03
Platelet Count (lakhs/cubic mm)	8.83 ± 0.08	6 ± 0.1	6 ± 0.2

All values are expressed in mean ± SEM (n = 6)

consumption during the study period. The status of bone marrow activity and intravascular effects were monitored by haematological examination as summarized [Table 3]. The administration of polymeric nanoparticles did not create any significant change in the levels of haemoglobin, RBC, polymorph and lymphocytes.WBC level slightly increased in PMMA polymer treated group. Nevertheless all values lay within normal limits. These results are considered as normal for this animal species. It is clear that liver and kidneys play significant role in various metabolic processes. Therefore, emphasis was placed on the effect these nanoparticles might have on the function of these organs. In addition, liver plays an important role in xenobiotic function; while kidneys are sites of reabsorption. Feeding of these nanoparticles did not alter the levels of glucose, protein, bilirubin, or creatinine indicating normal hepatocellular and nephrotic function [Table 2]. Serum AST and ALT levels remains normal and indicated that there is no leakage of enzymes from the hepatocytes and absence of hepatic necrosis. This is in consonance with the previous reports of PMMA nanoparticles toxicity studies.[9,19]

The evaluation of drug carriers also has to focus on the toxicity of such systems. Cationic carriers such as the polycations protamine, poly-L-lysine, and histone showed toxic effects in various cell culture systems, whereas cationized bovine serum albumin and diethy-laminoethyldextran, exhibited only little cytotoxicity.[20] In contrast, PMMA nanoparticles showed no cytotoxicity in rat hepatocytes as shown by electron microscopy and lactate dehydrogenase assay.[21] PLGA, a slow biodegradable polymer made up of L-lactic acid, a nontoxic, natural by- product of anaerobic metabolism of glucose.[22] Very recently,[23] researchers from Beijing chaoyang hospital, China, have reported the first case of the clinical toxicity of polyacrylate nanoparticles in humans. The main evidence involves, round nanoparticles ~30 nm in diameter were observed to lodge in the cytoplasm and karyoplasms of pulmonary epithelial and mesothelial cells and also in chest fluid. However, there are not enough data to strongly suggest that the clinical symptoms of the patient result from the toxicity of nanoparticles themselves. Therefore, adequate experimental data regarding toxicity is necessary for all the nanodrug delivery system. Our study demonstrated that oral administration of PMMA and PLGA nanoparticles loaded with metformin had fine biological compatibility without any toxic effects in the rats. The histopathologic effect of nanoparticles on the various organs such as heart, lung, liver and kidney were investigated after 30 days. The pathology changes of each organ were observed by light and electron microscopic measurements [Figure 4] exhibited the light microscopic images of each organ treated with Metformin loaded PMMA nanoparticles. No histopathologic changes were observed in treated groups compared with normal group as a control [Figure 4]. Accumulation of nanoparticles was not observed in the tissues pathology report indicating the non toxic nature in the pharmacokinetics of nanoparticle. But mononuclear cell infiltration was observed in kidney tissue and mild sinusoidal distension was observed in liver tissue.

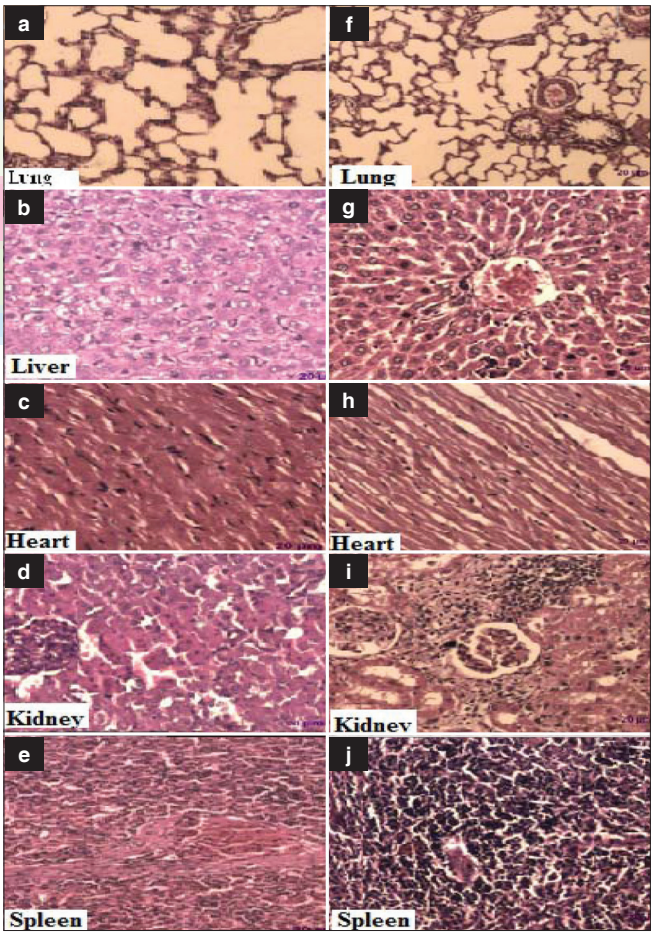


Figure 4: Organ tissue from rats exposed to Metformin loaded PMMA polymeric nanoparticle at a dose of 5 mg/kg body weight on 30 days post-oral administration (magnification = 200). Sections of the control animal compared with the treated animal for pathological examination. a-e slides were organs of control animals, f-j were organs of nanoparticle treated rats

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From histopathological analysis, it could be confirmed that drug loaded PMMA and 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. Therefore, it can be concluded that the PMMA and PLGA nanoparticles loaded with metformin 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. Further studies both *in vitro* and *in vivo* in details are required for more complete understanding and it's under progress.

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