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Research Paper

Development of Biodegradable Nanoparticles for Oral Delivery of Ellagic Acid and Evaluation of Their Antioxidant Efficacy Against Cyclosporine A-Induced Nephrotoxicity in Rats

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Purpose: Ellagic acid (EA), a dietary antioxidant associated with poor biopharmaceutical properties, was encapsulated into poly(lactide-co-glycolide) (PLGA) and polycaprolactone (PCL) nanoparticles to improve oral bioavailability.

Materials and Methods: EA-loaded nanoparticles were prepared following emulsion–diffusion–evaporation method employing didodecyldimethyl ammonium bromide (DMAB) and polyvinyl alcohol (PVA) as stabilizers. *In vitro* release was investigated in phosphate buffer (pH 7.4). The *in situ* permeation studies were performed in rats. The antioxidant potential of the DMAB-stabilized nanoparticulate formulations was evaluated against cyclosporine A (CyA)-induced nephrotoxicity in rats.

Results: EA-loaded PLGA and PCL nanoparticles have been succesfully prepared employing PEG 400 as co-solvent to solubilize EA. The stabilizers influenced the particle size and encapsulation efficiency. DMAB when used as stabilizer to particles of ~120 nm and ~50% encapsulation, whereas PVA led to ~290 nm and ~60% encapsulation at 5% initial loading (w/w of polymer). The *in vitro* release of EA from the nanoparticles followed Higuchi's square root pattern and was faster with PVA-stabilized particles in comparison to those stabilized with DMAB. From the *in situ* permeation studies in rats, it was evident that intestinal uptake of EA as DMAB-stabilized nanoparticles was significantly higher as compared to the sodium carboxymethyl cellulose suspension and the PVA-stabilized particles. EA and EA nanoparticles were able to prevent the CyA-induced nephrotoxicity in rats as evident by biochemical parameters as well as kidney histopathology.

Conclusion: The present study demonstrates the potential of EA nanoparticulate formulations in the prevention of CyA-induced nephrotoxicity at three times lower dose suggesting improved oral bioavailability of EA.

KEY WORDS: bioavailability; biodegradable; cyclosporine A; ellagic acid; free radicals; nanoparticles; nephrotoxicity; oral delivery.

This paper is dedicated to Ramesh C. Gupta, Professor and Agnes Brown Duggan Chair in Oncological Research, University of Louisville, US, who inspired me with his scientific approach, honesty and human warmth.

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ABBREVIATIONS: BCS, Biopharmaceutical classification system; BD, Bowman's capsule diameter; BUN, blood urea nitrogen; CD, capillary tuft diameter; CMC, carboxy-methyl cellulose; CyA, cyclosporine A; DMAB, Didodecyldimethyl ammonium bromide; EA, ellagic acid; EDE, emulsion-diffusion-evaporation; GIT, gastro-intestinal tract; mEDE, modified emulsion-diffusion-evaporation; PC, plasma creatinine; PCL, Polycaprolactone; PEG, polyethylene glycol; PLGA, poly(lactide-co-glycolide); PVA, polyvinyl alcohol; RP-HPLC, reversed phase high performance liquid chromatography; SD, Sprague Dawley; TBARS, thiobarbituric acid reacting substances.

INTRODUCTION

Free radicals and oxidative stress have been found to play a key role in many diseases and in this context, antioxidants have gained a lot of importance because of their potential as prophylactic and therapeutic agents in many diseases (1,2). Literature evidenced the role of free radicals in cancer, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders, aging and many other diseases (3–6). On the other hand, drugs like cisplatin, doxorubicin and CyA are known to induce oxidative stress that could further lead to chronic complications where simultaneous administration of antioxidants has been proven beneficial (7–9). These antioxidants also find applications in cosmetics, further fuelling research by industry and academia to explore these molecules and their analogues. Though not many antioxidants are listed in pharmacopoeias, extensive research is being carried out globally on these agents, and most of them have been proven pharmacologically active.

EA is a potent dietary antioxidant found in variety of fruits, nuts and many other food sources (10). It possesses a wide array of pharmacological activities like prevention and treatment of cancer, diabetic complications, atherosclerosis,

hypertension etc (11). EA also finds application in cosmetology as a skin whitening agent as it inhibits radiation induced melanogenesis. Reports are available on antibacterial and blood coagulating properties of EA; however, many of its activities are yet to be proven in humans. EA, with poor solubility (less than 10 μg/ml in phosphate buffer pH 7.4) and permeability (0.13×10^{-6}) can be classified as class IV drug under biopharmaceutical classification system (BCS) (12). Apart from poor solubility and permeability, EA have poor stability at physiological pH (13,14). Additionally, it is metabolized by intestinal microorganism upon oral administration and rapidly eliminated from the body due to short plasma half life (15,16). These biopharmaceutical and pharmacokinetic hurdles leads to poor oral bioavailability of EA and hindered its development beyond preclinical stage.

Nanoparticles have emerged as most promising delivery systems for such poorly bioavailable compounds and have been extensively studied in the past few years for oral delivery (17,18). The nanocarriers by virtue of their size and surface properties are taken up intact by M cells in Payer's patches of the gut associate lymphoid tissue followed by its systemic circulation (19). The nanocarriers can improve the oral bioavailability of poorly bioavailable drugs due to their specialized uptake mechanism. These polymeric nanocarriers are capable of preventing the gastro-intestinal degradation and first pass metabolism of encapsulated drugs (18,20). Furthermore, nanoparticles are capable of sustaining drug release in plasma for longer time period, thus reduce the frequency of administration. Biodegradable and non-degradable particles have been explored for this purpose, however, biodegradable carriers made of PLGA or PCL have an edge over others for the established safety of these materials (21). The present manuscript reports the development of PLGA and PCL nanoparticles containing EA and evaluation of their antioxidant potential against CyA-induced nephrotoxicity in rats.

MATERIALS AND METHODS

Materials

EA and PCL (Mol. Wt. 43,000–50,000) were purchased from Fluka (USA) and Polysciences (USA), respectively. PLGA (Resomer RG 50:50 H; inherent viscosity 0.41 dl/g) was a gift sample from Boehringer Ingelheim (Ingelheim, Germany). DMAB and PVA (Mol. Wt. 30,000–70,000) were purchased from Aldrich (St. Louis, MO, USA) and Sigma (St. Louis, MO, USA), respectively. Ethyl acetate (AR grade) and acetonitrile (ACN; HPLC grade) were purchased from Rankem Fine Chemicals (New Delhi, India). Ultrapure water (SG Water Purification System, Barsbuttel, Germany) was used for all the experiments. All the other chemicals and reagents were of highest commercially available grade.

Preparation of EA Loaded Nanoparticles

EA loaded nanoparticles of PLGA and PCL were prepared by adopting emulsion-diffusion-evaporation (EDE) method previously reported by our group for EA loaded PLGA-nanoparticles with slight modifications (mEDE) (22). Briefly, 50 mg PLGA or PCL was dissolved in 2.5 ml ethyl acetate by stirring at 40°C. EA (2.5 mg) was dissolved in 600 μl of PEG 400, which was then added to polymer solution and thoroughly mixed. This organic phase was then slowly emulsified with 5 ml of 1% w/v DMAB or PVA solution with stirring, followed by homogenization at 15,000 rpm for 5 min. To this, 30 ml of water was added and the preparation was stirred overnight at room temperature to remove the organic solvent. The nanoparticles were then separated by centrifugation at 8,000 rcf for 10 min and washed twice with distilled water to remove unbound stabilizer and unentrapped EA.

Characterization of Nanoparticles

The size of nanoparticles was determined by dynamic light scattering (Nano ZS, Malvern, UK), taking the average of five measurements, whereas zeta-potential was estimated on the basis of electrophoretic mobility under an electric field, taking an average of 30 measurements.

Encapsulation Efficiency

EA loaded nanoparticles were centrifuged and washed with 10 ml water. Then EA was extracted form the pellet with methanol by disrupting the nanoparticles using probe sonicator followed by estimation using a validated reverse-phase (RP) HPLC method (13). HPLC analysis was performed using Shimadzu Class LC-VP HPLC system with Class VP software, coupled to a UV detector (SPD-10Avp) (Shimadzu, Japan). The separation was achieved on the Supelco Discovery® HS polyethylene glycol (PEG) column, 25 cm×4.6 mm, 5 μm (Supelco, Bellefonte, PA, USA) maintained at 40°C. EA was eluted isocratically at a flow rate of 1 ml/min using mobile phase consisting of 5 mM potassium dihydrogen orthophosphate buffer (pH 2.5) and acetonitrile (20:80 v/v). Absorbance of eluent was measured at 254 nm and the retention time for EA was 4 min.

In Vitro Release

In vitro release of EA from the nanoparticles was determined by the dialysis membrane method. The nanoparticles containing 1 mg of EA suspended in 1 ml of phosphate buffer (pH 7.4) and transferred to dialysis bag (molecular mass cut-off 12,000 Da). The dialysis bags were placed in glass vial containing 5 ml of phosphate buffer and maintained at 37°C in shaker bath. At predetermined time intervals, the release medium was completely replaced with fresh medium. The amount of EA released was analyzed by validated RP-HPLC method.

In Situ Permeation Study

The intestinal uptake of the free EA (as sodium CMC suspension) and EA loaded nanoparticles was assessed using closed loop method as previously reported by our group (18). All animal experiments were performed according to a protocol duly approved by the Institutional animal ethics committee (IAEC) of NIPER. Briefly, overnight fasted male Sprague Dawley (SD) rats weighing between 200–250 g were

anaesthetized by intraperitoneal administration of thiopentone sodium (50 mg/kg). The intestine was exposed through a midline incision on the abdomen and a closed loop of 10 cm length was prepared on the upper jejunum by ligation at the both ends. 1 ml of aqueous EA suspension or EA loaded nanoparticles (dose 500 μg) was injected into the loop with a syringe. After 2 h, the loops were isolated from the body and loop contents were emptied into a volumetric flask. The lumen was washed with 25 ml methanol and the whole content was sonicated for 20 min followed by centrifugation at 25,000 rcf for 10 min. The clear supernatant was then analyzed by validated RP-HPLC method for drug content.

In Vivo Evaluation of Antioxidant Potential of DMAB Stabilized EA Nanoparticles Against CyA-Induced Chronic Nephrotoxicity

Male SD rats, weighing 200–250 g were used for the study. They were housed, three per cage and kept on 12/12 h light/dark cycles with controlled temperature (20–22°C). The animals were provided with normal pellet diet and free access to water during whole period of experimentation. The animals were randomly distributed into seven groups, each comprising of three animals. Treatment was started on day one as stated in Table I. Group-I was kept as control without any treatment. Group-II received a daily dose of 15 mg/kg of CyA in the form of Sandimmune Neoral® (commercial formulation of CyA) for 30 days. Along with the CyA treatment (15 mg/kg/day), Group-III received daily 50 mg/kg of EA as a suspension in 0.5% w/v sodium carboxymethyl cellulose (CMC) vehicle; Group-IV was treated with EA loaded PLGA-DMAB nanoparticles at a dose of 50 mg/kg every third day; Group-V received EA loaded PCL-DMAB nanoparticles at a dose of 50 mg/kg every third day; Group-VI and VII were administered with blank PLGA-DMAB nanoparticles and PCL-DMAB nanoparticles equivalent to that of EA loaded nanoparticles, respectively. The animals were fasted 6 h before all the treatments.

Sample Collection and Tissue Preparation

On 31st day, animals were sacrificed by decapitation under ether anesthesia and blood was collected in heparinized centrifuge tubes. Plasma was separated by centrifugation at 6,000 rcf for 5 min and analyzed for the biochemical parameters including blood urea nitrogen (BUN), plasma creatinine (PC) and malondialdehyde (MDA) levels in kidneys and plasma.

Assessment of Renal Function

The renal function in experimental groups was assessed by estimating the BUN and PC levels using the commercially available kits (Accurex Biomedical Pvt. Ltd, Mumbai, India).

Assessment of Lipid Peroxidation

MDA is a measure of lipid peroxidation and was assayed in kidneys and plasma in the form of thiobarbituric acid reacting substances (TBARS) according to the reported method (23). In brief, the reaction mixture consisted of 0.1 ml of 8.1% sodium lauryl sulphate, 0.75 ml of 20% acetic acid solution adjusted to pH 3.5 and 0.75 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.1 ml of plasma sample or tissue homogenate and the volume was adjusted to 2 ml with distilled water followed by heating at 95°C for 60 min. After cooling, the mixture was centrifuged and the supernatant was analyzed by measuring its absorbance at 532 nm. The amount of TBARS formed was calculated against the calibration curve prepared using MDA as standard.

Histopathological Evaluation of Kidneys

The right kidneys were isolated immediately after sacrificing the animal. The tissues were washed with ice-cold saline followed by its fixation in 10% neutral buffered formalin solution. The tissues were embedded in paraffin blocks and 5 μm thick sections were cut, deparaffinized, hydrated and stained with hematoxylin and eosin. The histological sections were examined under microscope (Leitz, Wetzlar, Germany) for the morphological changes in all treatment groups. The diameters of the glomerular capillary tuft (CD) and the Bowman's capsule (BD) were measured with the help of internal micrometer and the ratio CD/BD calculated as an index of glomerular collapse (36).

Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by Tuckey's multiple comparison test for comparing means from different treatment groups. The data were expressed as mean ± S.E.M. and a value of $P<0.05$ was considered statistically significant.

Table I. Study Plan for Nephrotoxicity

Experimental Group	Sandimmune Neoral® Dosing (15 mg/kg/day)	EA Dosing Frequency (50 mg/kg)
Group-I (Control)	–	–
Group-II (Sandimmune Neoral®)	✓	–
Group-III (EA suspension in sodium CMC)	✓	Daily
Group-IV (EA loaded PLGA-DMAB nanoparticles)	✓	Every 3rd day
Group-V (EA loaded PCL-DMAB nanoparticles)	✓	Every 3rd day
Group-VI (Blank PLGA-DMAB nanoparticles)	✓	–
Group-VII (Blank PCL-DMAB nanoparticles)	✓	–

Table II. Effect of Stabilizer on EA Loaded Nanoparticles of PLGA and PCL

	PLGA		PCL	
	DMAB	PVA	DMAB	PVA
Particle size (nm)	125.2±4.8	293±7.53	128±8.08	281±7.53
PDI	0.278±0.02	0.178±0.1	0.281±0.01	0.121±0.03
Zeta potential (mV)	78.5±5.78	−7.09±0.98	63.5±6.83	−5.09±1.75
% EE	52.1±4.8	61.56±5.2	47.4±5.69	57.3±8.6

All values are mean ± S.D. (n=3)
PDI: polydispersity index, % EE: percent encapsulation efficiency.
The zeta potential values reported are in the pH range 3.95–4.83 for particles with DMAB and 5.23–5.79 for particles with PVA as stabilizer.

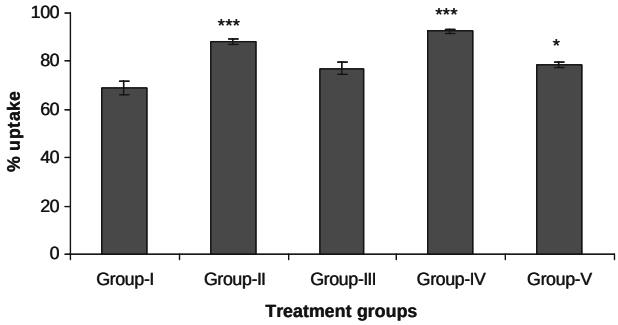


Fig. 2. *In situ* intestinal permeation of EA as suspension and its nanoparticulate formulations. Values expressed as Mean ± S.E.M. (n=3), ***p<0.001 and *p<0.05 as compared to EAS. Treatment groups: Group-I: EA suspension in sodium CMC. Group-II: EA loaded PLGA-DMAB nanoparticles. Group-III: EA loaded PLGA-PVA nanoparticles. Group-IV: EA loaded PCL-DMAB nanoparticles. Group-V: EA loaded PCL-PVA nanoparticles.

RESULTS AND DISCUSSION

Preparation of EA Loaded Nanoparticles

The choice of a particular method for preparation of drug loaded nanoparticles is most commonly determined by the solubility characteristics of the drug as well as the polymer. PLGA and PCL being soluble in organic solvents, their nanoparticles were formed using o/w emulsion technique. The basic EDE methodology involves the preparation of a stable primary emulsion by stirring at 1,000 rpm for 3 h followed by its homogenization, but we found that nanoparticles can be prepared without going through the primary emulsion step, we called it mEDE method. The reason for investigating this was the rapid precipitation of EA in polymer solution during primary emulsion step, which led to its low encapsulation efficiency. The omission of primary emulsion step in mEDE led to higher encapsulation efficiencies without compromising the particle size (Table II). Moreover, mEDE method was rapid and robust compared to EDE as the polymer solution was directly homogenized with stabilizer solution minimizing the possibility of errors that could result in due to variation in the rate of addition, stirring speed and time.

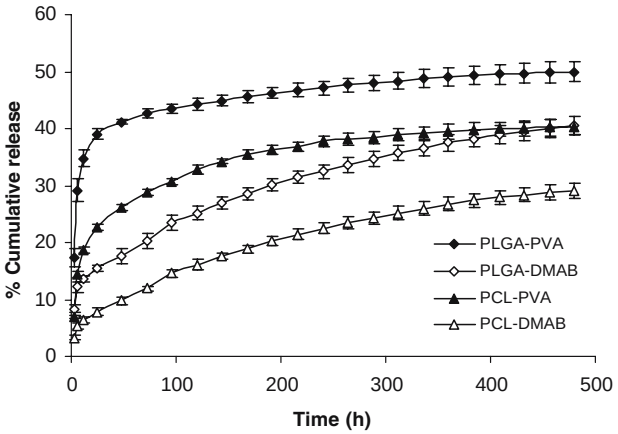


Fig. 1. *In vitro* release profile of PLGA and PCL nanoparticles in phosphate buffer (pH 7.4) values expressed as Mean ± S.D. (n=3).

Both the EDE and mEDE methods allowed easy and reproducible preparation of EA loaded nanoparticles using PLGA and PCL where mEDE method led to higher encapsulation efficiencies as compared to EDE method.

The type of stabilizer has significant effects on the overall particle characteristics. The particle size was clearly dependant on the type of stabilizer used rather than type of polymer. Irrespective of polymer type, use of DMAB as stabilizer led to much smaller sized particles as compared to PVA (Table II). Since, nanoparticle preparation by EDE techniques involves stabilization of the emulsion by stabilizers, it can be stated that better the ability of a stabilizer to lower the interfacial tension between aqueous and organic phases, smaller would be the particle size. The ability of a stabilizer to lower interfacial tension is reflective of the capacity of its hydrophobic portion to bind to the organic phase droplet and its hydrophilic portion to remain saturated with aqueous phase (24). It was thought that long hydrophobic chain of DMAB binds to the surface of organic phase droplet with higher binding constant in comparison to more hydrophilic PVA chains, making DMAB more efficient in lowering the interfacial tension with resultant formation of smaller particles (18).

The type of stabilizer also had significant effect on the zeta potential of the nanoparticles that could further influence the particle performance. Since DMAB is a cationic stabilizer, it led to +ve zeta potential of around 65–70 mV, on the other hand, PVA led to -ve zeta potential of −3.5–−7.5 mV (Table II). The encapsulation efficiency of EA was also found to be influenced by the nature of the stabilizer used. The PVA stabilized particles resulted in 57–61% encapsulation, whereas DMAB stabilized particles led to 47–52% encapsulation efficiency at 5% initial loading (w/w of polymer). The possible reason for this difference could be the solubility of EA in the surfactant solutions that needs further investigation.

In Vitro Release Study

Drug release from nanoparticles is interplay of various factors including nature of polymer, physicochemical prop-

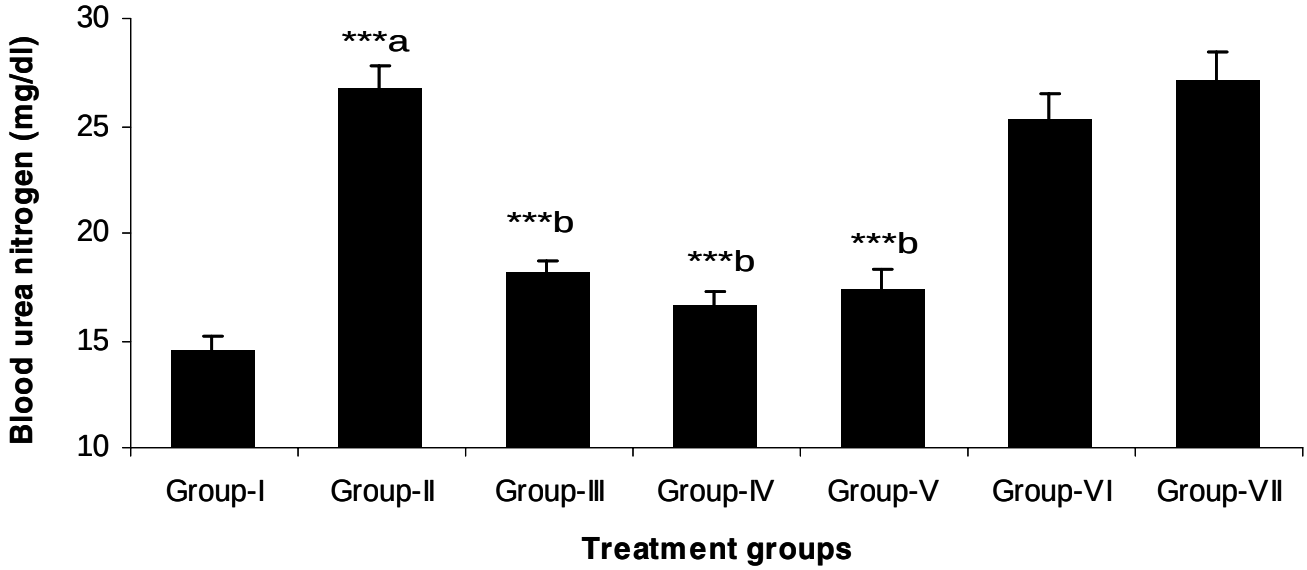


Fig. 3. Blood urea nitrogen (BUN) levels in rats on 31st day of study, each point is represented as Mean ± S.E.M., ***P<0.001, a vs control, b vs Sandimmune Neoral® (n=3). Treatments: Group-I: Control (No treatment). Group-II: Sandimmune Neoral®. Group-III: EA suspension + Sandimmune Neoral®. Group-IV: EA loaded PLGA-DMAB nanoparticles + Sandimmune Neoral®. Group-V: EA loaded PCL-DMAB nanoparticles + Sandimmune Neoral®. Group-VI: Blank PLGA-DMAB nanoparticles + Sandimmune Neoral®. Group-VII: Blank PCL-DMAB nanoparticles + Sandimmune Neoral®.

erties of drug, type of stabilizer and the formulation factors. The drug release from the biodegradable nanoparticles occurs through several mechanisms such as desorption of surface adsorbed drug, disintegration and diffusion of drug from nanoparticle matrix and finally through degradation of polymer matrix.

The cumulative release profiles of EA from PLGA and PCL nanoparticles prepared using PVA and DMAB are shown in Fig. 1. The cumulative drug release was fitted into different release models namely zero order, first order, Higuchi's square root plot and Hixson–Crowell cube root plot and the model giving a correlation coefficient close to unity was taken as order of release. An initial burst release was found in all the formulations, followed by Higuchi's square root pattern with r^2 values of 0.9854 and 0.9146 in case of PVA stabilized PLGA and PCL nanoparticles, while 0.9913 and 0.9908 for DMAB stabilized PLGA and PCL nanoparticles, respectively.

The nanoparticles prepared using PVA as stabilizer showed fast initial release with about 35% (PLGA) and 23% (PCL) of total drug being released within 24 h, whereas the DMAB stabilized nanoparticles showed less initial release, with about 15 and 8% for PLGA and PCL, respectively. Thus, the release mechanism can be best described by diffusion alone during the initial phase followed by combined diffusion and degradation in the later phases. The cosolvent PEG 400 could be playing a significant role in the release behavior. The particles stabilized by PVA resulted in higher encapsulation efficiency in comparison to DMAB stabilized particles leading to increased PEG 400 concentration in the particles, which may subsequently leave more voids leading to faster release.

Literature evidenced that the type of polymer used can influence the release behavior significantly and reports specifically indicate PCL is slower degrading polymer in comparison to PLGA thereby leading to slower release. However, this can be further dependent on various other parameters as can be seen in the present case. PCL-DMAB nanoparticles showed the lowest release, whereas PLGA-PVA nanoparticles showed faster and higher release despite of their larger size where both polymer as well as encapsulation efficiency playing a role, which is facilitated by PEG 400.

In Situ Permeation Study

In situ permeation studies were performed to evaluate the absorption enhancing potential of the nanoparticles. The advantage of the *in situ* system compared to the *in vitro*

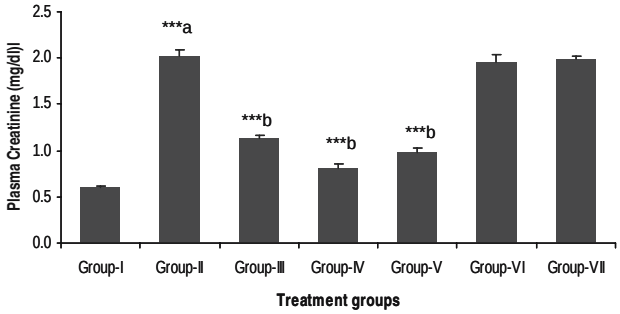


Fig. 4. Plasma Creatinine (PC) levels in rats on 31st day of study, each point is represented as Mean ± S.E.M., ***P<0.001, a vs. control, b vs. Sandimmune Neoral® (n=3). Treatment groups: Group-I: Control (No treatment). Group-II: Sandimmune Neoral®. Group-III: EA suspension + Sandimmune Neoral®. Group-IV: EA loaded PLGA-DMAB nanoparticles + Sandimmune Neoral®. Group-V: EA loaded PCL-DMAB nanoparticles + Sandimmune Neoral®. Group-VI: Blank PLGA-DMAB nanoparticles + Sandimmune Neoral®. Group-VII: Blank PCL-DMAB nanoparticles + Sandimmune Neoral®.

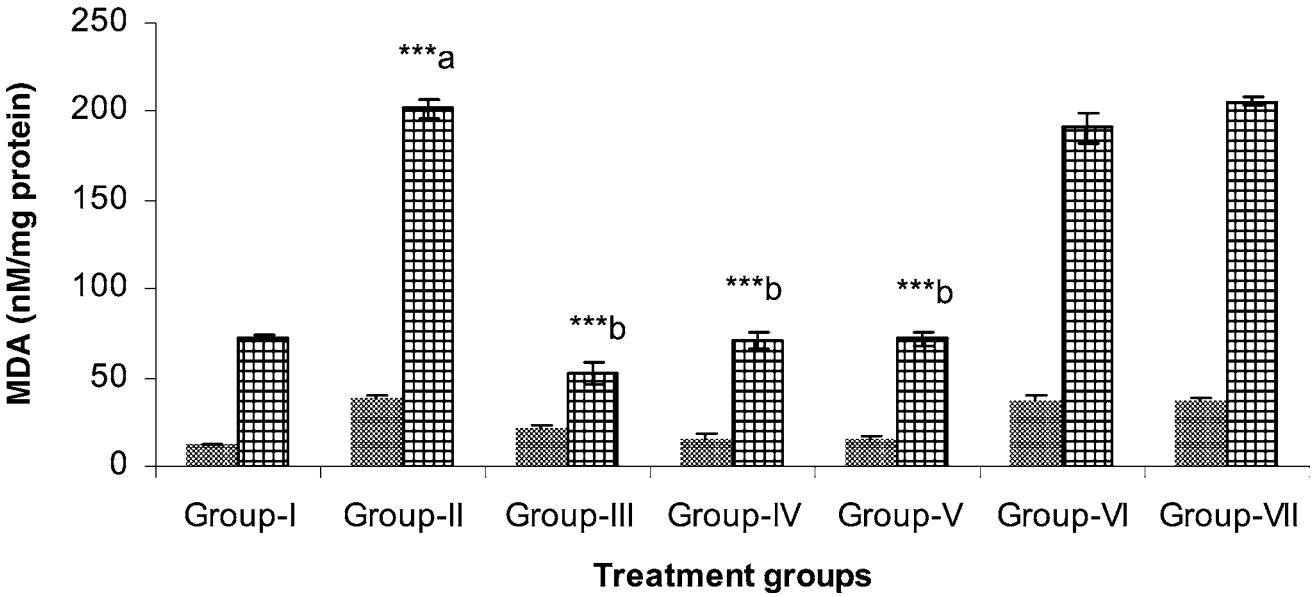


Fig. 5. Plasma and kidney MDA levels in rats on 31st day of study, each point is represented as Mean ± S.E.M., ***P<0.001, a vs. control, b vs. Sandimmune Neoral® (n=3). Treatment groups: Group-I: Control (No treatment). Group-II: Sandimmune Neoral®. Group-III: EA suspension + Sandimmune Neoral®. Group-IV: EA loaded PLGA-DMAB nanoparticles + Sandimmune Neoral®. Group-V: EA loaded PCL-DMAB nanoparticles + Sandimmune Neoral®. Group-VI: Blank PLGA-DMAB nanoparticles + Sandimmune Neoral®. Group-VII: Blank PCL-DMAB nanoparticles + Sandimmune Neoral®.

techniques is the presence of an intact blood and nerve supply in the experimental animals. The uptake of EA was assessed based on the disappearance of drug from the intestinal lumen. The particle uptake is reported to be the size dependent phenomena, where the small sized particles could be efficiently taken up as compared to the bigger sized particles (18,24). Apart form the size, surface charge of the particles also plays crucial role in the particle uptake. It has been reported that the electrostatic interaction between the mucosal surfaces and the particulate carriers has a significant effect on the particle uptake and thus overall bioavailability (25).

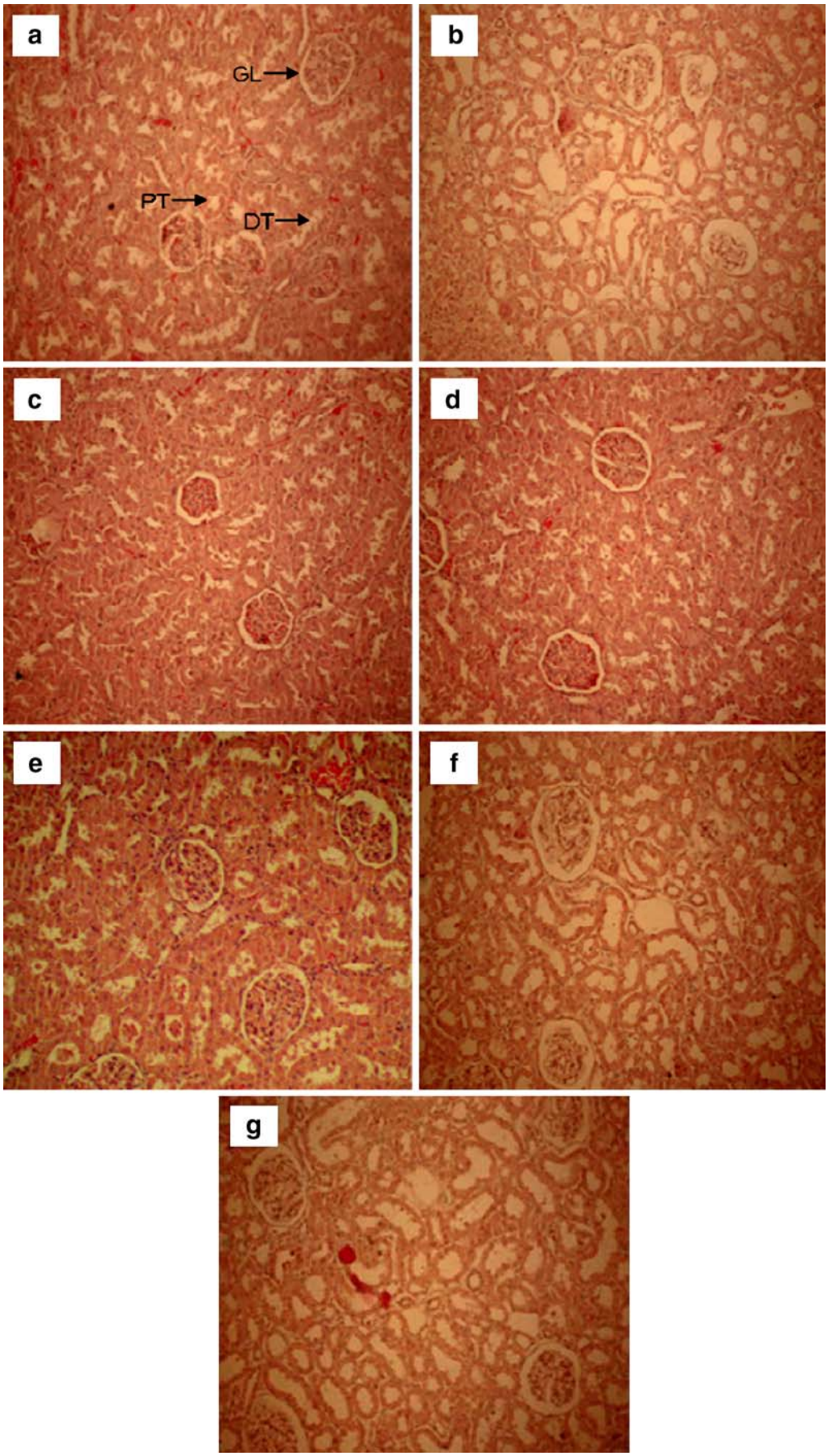
Among the EA loaded PLGA and PCL particles, the positively charged DMAB stabilized particles showed significantly higher (P<0.001) uptake in comparison to negatively charged PVA stabilized particles. EA in the form of sodium CMC suspension showed significantly lower (P<0.001) uptake of around 68% as compared to the DMAB stabilized PLGA and PCL particles, which showed around 87 and 93% uptake, respectively. PVA stabilized PLGA and PCL nanoparticles showed nearly equal intestinal uptake of 77 and 78%, respectively, which was slightly higher then the EA suspension (Fig. 2).

Since, DMAB stabilized particles are cationic in nature, it facilitates electrostatic interaction with negatively charged mucosa leading to the improved particle uptake. Apart from cationic nature, the small size of the DMAB stabilized particle also account for its enhanced uptake in comparison to the nanoparticles made with PVA. Moreover, it has been observed that a fraction of unbound PVA in the particle preparation often poses problems in the cellular uptake of the particles (26). Since free EA has poor permeability and is prone to degradation in gastro-intestinal tract (GIT), the 68% uptake for simple suspension might not reflect the actual uptake of the EA, rather part of it could have been degraded.

In Vivo Evaluation of Antioxidant Potential of DMAB Stabilized EA Nanoparticles Against CyA-Induced Chronic Nephrotoxicity

Cyclosporine is an immunosuppressive agent used for prevention of graft rejection following organ transplantation and in the treatment of autoimmune disorders but its clinical use is limited by adverse effects, the most important of which is nephrotoxicity. Chronic CyA nephrotoxicity is manifested by renal insufficiency due to glomerular and vascular disease in addition to the abnormalities in tubular function. The molecular mechanism of CyA-induced nephrotoxicity is still a matter of debate. However, numerous studies have been reported suggesting that CyA-induced toxicity could be due to the consequence of oxidative stress leading to oxidation and cross-linking of cellular thiols and membrane lipid peroxidation. After discovery of the role of oxidative stress in pathogenesis of CyA-induced nephrotoxicity, many attempts have been made to prevent its side effects by co-treatment with antioxidants. The exogenous antioxidants like vitamin E, vitamin C, epigallocatechin gallate and resveratrol

Fig. 6. (a) renal cortex of control (untreated) rat showing normal ▶ glomeruli (GL), proximal tubule (PT) and distal tubule (DT); (b) CyA (Sandimmune Neoral®) treated rats showed characteristic morphological changes including wide spread tubular atrophy and glomerular collapse resulted from ischemic atrophy of the glomerular tuft; (c) Concomitant administration of EA as suspension largely prevented such morphological alterations; (d) and (e) co-treatment with both, EA loaded PLGA-DMAB and PCL-DMAB nanoparticles, provided similar kind of protection from the CyA-induced renal damage at three times lower dose; (f) blank PLGA-DMAB nanoparticles and (g) blank PCL-DMAB nanoparticles treatment along with CyA showed the similar kind of morphological alteration as observed in CyA treated rats (Hematoxyline and eosin staining, X 200).


a
GL
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have been reported to be effective in preventing nephrotoxicity of CyA (27–29). However, most of these studies were done with the parentral administration due to the unfavorable biopharmaceutical properties and low oral bioavailability of most of the antioxidants. Thus, present study was conducted to improve oral bioavailability of EA by encapsulating the same into nanoparticles.

Chronic CyA-induced nephrotoxicity is characterized by increased BUN, PC and MDA levels in kidney and plasma. BUN and PC are important biochemical parameter, which are routinely evaluated as an indicator of renal function. Both, BUN and PC are filtered by the kidneys and abnormal rise in their blood levels indicate renal dysfunction or damage.

Chronic CyA (Sandimmune Neoral®) treatment caused significant ($P<0.001$) increase in BUN levels as compared to control rats. Concomitant treatment with EA as sodium CMC suspension significantly ($P<0.001$) prevented the increase in the BUN level, indicating its protective effect (Fig. 3). Concomitant treatment with EA as nanoparticulate formulations (both PLGA and PCL) were found to be more efficient than the suspension in preventing the CyA-induced renal damage at three time reduced dose.

Similarly, chronic CyA treatment also led to significant ($P<0.001$) rise in the PC levels as compared to control group again conforming nephrotoxicity in that group. Concomitant treatment with EA as suspension and nanoparticulate formulations significantly ($P<0.001$) prevented increase in PC levels, where both the nanoparticulate formulations again showed higher activity than EA suspension at three times lower dose (Fig. 4).

A close relationship between oxidative stress and CyA-induced nephrotoxicity has been conformed in many experiments (30,31). CyA treatment has been reported to increase MDA, which is a stable product of lipid hydro-peroxide in isolated hepatic microsomes and mesangial cells (32). This observation suggested that CyA or its metabolites produce free radical species that attack lipid components, leading to lipid peroxidation.

Consistent with this possibility, the data obtained showed a significant ($P<0.001$) increase in plasma and kidney MDA levels following chronic CyA treatment, again suggesting the involvement of oxygen free radicals in the pathogenesis of renal injury during CyA treatment (Fig. 5). Concomitant administration of both, EA suspension and EA loaded nanoparticles led to significantly ($P<0.001$) lower MDA levels in plasma and kidneys indicating the protective effect of EA against CyA-induced oxidative stress. The nanoparticulate formulations were again found to be more efficacious in comparison to the suspension at three times reduced dose. The blank nanoparticles of both PLGA and PCL neither attenuate nor accelerated the CyA-induced nephrotoxicity as indicated by similar BUN, PC and MDA levels in the plasma and kidneys, which showed no difference as compared the control group indicating their inertness.

The results obtained (Figs. 3, 4 and 5) clearly indicated the improved bioavailability of EA in the form of nanoparticulate formulations. Prevention of degradation of EA in the gastro-intestinal tract along with the enhanced intestinal uptake and protection from first pass metabolism by nanoparticulate carriers could contribute to its improved oral bioavailability. Interestingly, both PLGA and PCL formulations are found to be equally effective leaving a possibility for developing a cost effective formulation using PCL, which is considerably cheaper when compared to PLGA.

Histopathological Evaluation of Kidneys

Structural changes, which are associated with chronic CyA-induced nephrotoxicity includes diffuse or stripped interstitial fibrosis with tubular atrophy, obliterate arteriolopathy and ischemic atrophy of the glomerular tuft (glomerular collapse) (33–36). These morphological damage leads to progressive loss of renal functions.

The renal histopathological changes following chronic CyA treatment alone and concomitant administration of EA as a suspension and DMAB stabilized PLGA and PCL nanoparticles is shown in Fig. 6. The morphometric analysis of histological specimens is shown in Table III. The light microscopic findings of kidneys of control rats (without treatment) showed normal glomeruli as well as proximal and distal tubules (Fig. 6a). By contrast, the kidneys of rats treated with CyA (Sandimmune Neoral®) showed significantly higher glomerular collapse ($P<0.001$ vs. control, Table III) and wide spread tubular atrophy (Fig. 6b). Concomitant administration of EA as suspension largely attenuated such morphological damage (Fig. 6c) with a significantly lower glomerular collapse ($P<0.01$ vs. Sandimmune Neoral®, Table III). Interestingly, concomitant administration of EA loaded nanoparticles (both PLGA and PCL) significantly ($P<0.001$ vs. Sandimmune Neoral®) reduced glomerular collapse and provided similar kind of the renal protection at three times lower doses (Fig. 6d and e). Concomitant treatment with blank nanoparticles, both PLGA and PCL, showed similar glomerular collapse and tubular atrophy as observed in the case of CyA treated rats, indicating that blank nanoparticles it self doesn't have any antioxidant activity (Fig. 6f, g and Table III).

Table III. Morphometric Analysis of Histological Sections of Kidneys

Experimental Group	Morphometric Analysis (CD/BD)
Group-I (Control)	0.893±0.022
Group-II (Sandimmune Neoral®)	0.764±0.002[a]
Group-III (Sandimmune Neoral® + EA suspension in sodium CMC)	0.845±0.013[b]
Group-IV (Sandimmune Neoral® + EA-PLGA-DMAB nanoparticles)	0.863±0.014[c]
Group-V (Sandimmune Neoral® + EA loaded PCL-DMAB nanoparticles)	0.865±0.021[c]
Group-VI (Sandimmune Neoral® + Blank PLGA-DMAB nanoparticles)	0.762±0.018
Group-VII (Sandimmune Neoral® + Blank PCL-DMAB nanoparticles)	0.765±0.023

All Values are mean ± S.E.M.
$P<0.001$, a *vs.* control and c *vs.* Sandimmune Neoral®, $P<0.01$ b *vs.* Sandimmune Neoral®.

CONCLUSION

The results revealed that EA can be efficiently entrapped into PLGA and PCL nanoparticles for oral delivery and these particles were able to sustain the release over a period of 20 days *in vitro*. The intestinal uptake studies in the animals suggested that nanoparticles can improve oral absorption, where the uptake was size and surface property dependent. Both, DMAB stabilized PLGA and PCL nanoparticles of EA, were more efficient in reducing the CyA-induced nephrotoxicity as compared to EA suspension at three times reduced dose indicating improved bioavailability of EA as nanoparticulate formulations. Literature has evidenced the role of EA in several diseases, so it would be quite interesting to see the potential of these formulations in prophylaxis and treatment of diseases like cancer.

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