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Targeting tacrolimus to deeper layers of skin with improved safety for treatment of atopic dermatitis—Part II: *In vivo* assessment of dermatopharmacokinetics, biodistribution and efficacy

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ABSTRACT

The objective of present investigation was to study *in vivo* behavior of tacrolimus-loaded lipid-nanoparticles (T-LN) to understand its targeting potential for treatment of atopic-dermatitis-(AD). T-LN have shown significantly improved drug penetration to deeper epidermal and dermal skin-layers than commercial ointment-Protopic® and effectively reached target dendritic-immune-cells, responsible for immunopathogenesis of AD. Due to enhanced penetrability of T-LN, it became necessary to evaluate the toxicity of the nanocarrier and the drug at non-target tissues. This paper evaluates dermatopharmacokinetics (DPK), biodistribution, efficacy and safety of T-LN in comparison to Protopic® as reference. *In vivo* DPK in guinea pigs showed 3.02-fold higher bioavailability while γ-scintigraphy in albino-rats demonstrated 1.5-fold rapid penetration of radioactivity in skin for T-LN. Biodistribution in albino-rats revealed restricted localization at the target-skin-area with no general spreading to other body organs suggesting targeting potential of T-LN. *In vivo* efficacy studies in BALB/c mice showed highly efficient suppression of inflammatory AD-like skin-lesions with T-LN than reference and placebo. Dermal toxicity-studies revealed keratosis and collagenous mass-infiltration with repeated application of reference however interestingly, T-LN treated group showed no evident toxicity demonstrating significantly improved safety. Thus T-LN offered improved penetration to the target site without any toxic-effects and would represent an efficient and commercially viable alternative for AD treatment.

1. Introduction

Atopic dermatitis (AD) is a complex disease that manifests immunological abnormalities in the skin and represents one of the most common chronically relapsing inflammatory skin diseases accompanied by severe itching and eczematous skin lesions (Kaltoft et al., 1994; Lee et al., 2000; Zheng and Zhu, 2005). Incidence of AD has increased in recent years in industrialized countries, and it often relapses after the termination of therapeutic treatment period (Granlund et al., 1995; Pivarcsi, 2004). Even though considerable efforts are marshaled against AD, it continues to recur throughout the patient's lifespan and inflict an unacceptable burden on many developing countries. The treatment of local dermatological disease condition by directly applying a pharmaceutical formulation to the site is easy, convenient and well accepted by patients. Topical corticosteroids are routinely used to manage AD; however, its use is limited to shorter duration due to adverse side effects such as easy bruising of skin, telangiectasia, allergy, increased susceptibility to skin infection, skin thinning and atrophy (Furue et al., 2003; Yamamoto and Nishioka, 2003).

Tacrolimus, a macrolide immunosuppressant drug, has been found to be useful in the treatment of many immune mediated dermatoses including AD (Yoshida et al., 2004; Rubins et al., 2005). Unlike the conventional therapy which involves use of topical corticosteroids; use of topical tacrolimus does not produce significant side effects. Tacrolimus is therapeutically effective in patients having sensitive skin, infants, children and elderly where steroids are likely to cause many side effects (Ruzicka et al., 1999). Tacrolimus achieves immunosuppression mainly by inhibition of interleukin 2 (IL-2) transcription thereby preventing T lymphocyte activation (Denise and William, 2005; Sehgal et al., 2008). Although being effective, topical application of tacrolimus is associated with burning sensation, itching or stinging along with other potential local side effects (Zahir et al., 2001; Hultsch et al., 2005; Rubins et al., 2005; Spergel and Leung, 2006; Ständer et al., 2006).

At present tacrolimus is available commercially as an ointment formulation, Protopic® for topical application. However, ointments

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are difficult to wash off the skin and due to their greasy nature may create a sticky feeling and uneasiness to the patient (Kudla, 1979; Lin et al., 2005). In addition, several studies with tacrolimus ointment have reported notably variable absorption rates and high individual variation in mean disposition half-life values (Ruzicka et al., 1997; Cheer and Plosker, 2001; Kang et al., 2001; Paller et al., 2001). Patients have experienced sensation of warmth and stinging together with pain and redness during clinical trials of the ointment (Ständer et al., 2006; Svensson et al., 2011). Allergic reaction, fever, flu-like symptoms and skin infection have also been reported (Wijnen et al., 1992; Zahir et al., 2001; Spergel and Leung, 2006). Systemic exposure to tacrolimus can be even more catastrophic. It may cause hives, difficulty in breathing, swelling of face, lips, tongue, or throat and increased susceptibility to bacterial, viral, fungal, and protozoal infections including opportunistic infections. Side effects can be severe with increased long-term malignancy risk and serious drug interactions (Zahir et al., 2001; Spergel and Leung, 2006). It has also been reported to have a potential to increase the risk of development of lymphoma and other malignancies, particularly of the skin, due to immunosuppression. There are reports of lymphoproliferative changes in rodents and certain cynomolgus monkeys after tacrolimus use (Wijnen et al., 1992). This toxicology data suggest an increased risk with tacrolimus use in cases of extensive and/or prolonged use. In order to overcome these disadvantages, to enhance the bioavailability at the target site and reduce the related toxicity it is necessary to exploit delivery of tacrolimus using novel formulation approaches with significantly improved beneficial impact for treatment of AD.

A major problem associated with drug therapy is the inability to deliver the pharmaceutical to a specific site of the body without causing any side effects. The advantages of topical application are mellowed by protective barriers of skin, which limit drug penetration when formulations are applied topically to the skin. Therefore the attention of different research groups has focused on novel nanotechnology based delivery systems which hold great promise to improve the efficiency and enhance bioavailability of drugs (Couvreur et al., 1995; Mühlen et al., 1998; Barratt, 2000; Jenning et al., 2000; Maia et al., 2000; Porter and Charman, 2001). The smaller size and unique properties of the nanoparticles have substantially improved their application in drug delivery (Pople and Singh, 2006, 2011; Schäfer-Korting et al., 2007; Bhalekar et al., 2009; Nagi et al., 2008; Goebela et al., 2011). Studies have shown that nanoparticles can pass through the protective barriers of living organisms and accumulate at specific sites. The corollary is that nanoparticles could be distributed throughout the body, which may lead to undesirable toxic effects mainly because of their enormous surface area. The area of toxicology, known as nanotoxicology, reports deleterious effects due to substances which are ordinarily innocuous but can have adverse effects at the nanoscale (Service, 2004; Dechsakulthorn et al., 2007; Wani et al., 2011). Toxic effects due to nanoparticles have been documented at the pulmonary (Bartel et al., 2011), cardiac (McLeish et al., 2010), renal (Lei et al., 2008), cutaneous (Choksi et al., 2010) and cellular levels (Pakrashi et al., 2011). Effects on reproduction (Cañas et al., 2011) and genotoxic effects (Kumari et al., 2011) have also been identified so far. These documented toxic effects of nanoparticles on animals justify the urge for application of all useful measures to effectively ascertain the potential hazards of these delivery systems.

Although few novel approaches such as liposomal tacrolimus lotion (Erdogan et al., 2002), colloidal carrier system ME (Goebela et al., 2011) and modified nanolipid carrier (Pople and Singh, 2011) have been investigated for topical tacrolimus delivery, none of these have been subjected to thorough assessment of systemic biodistribution and safety evaluation.

In our previous report (Pople and Singh, 2010), tacrolimus loaded lipid nanoparticles (T-LN) were developed, optimized for process-product variables and characterized in detail for *in vitro* parameters including drug release and skin penetration. We found that T-LN significantly improved drug penetration to the deeper epidermal and dermal layers of the skin as compared to commercially available product Protopic® and effectively reached the target dendritic immune cells that are responsible for immunopathogenesis of AD. Due to enhanced penetrability of T-LN, it became necessary to evaluate the risk associated with application of the nanocarrier (*i.e.* nanotoxicity) and toxicity related to drug itself at non-target tissues. This paper evaluates the pharmacokinetics, pharmacodynamics and safety of T-LN in comparison to the commercially available ointment product Protopic® as reference. The bioavailability of T-LN was assessed employing dermatopharmacokinetic (DPK) approach in guinea pigs and bio-deposition was measured using gamma scintigraphy in albino rats. It is crucial that the drug reaches its specific site of action but it is equally important that it does not distribute to other organs of the body causing drug-related toxic effects. This becomes even more important when the drug is being applied topically and systemic toxicity needs to be avoided. Biodistribution studies were carried out using gamma scintigraphy in albino rats to confirm target specific delivery. Therapeutic efficacy was appraised using a hapten-induced murine model of AD in BALB/c mice. In addition, acute and repeated dose dermal toxicity studies (OECD 402 and 410) were performed in order to establish detailed safety profile. All experimental procedures involving animal use were reviewed and approved by Institutional Animal Ethics Committee.

Table 1
The composition of T-LN.

Tacrolimus	0.1%
Trimyristin	5 g
Sorbitan monooleate	3.5 g
Polyoxyethylene sorbitan monooleate	4.0 g
Double distilled water	q.s. 100 g

2. Materials

Tacrolimus was procured from Panacea Biotec Ltd., Punjab, India. Glyceryl trimyristate (Dynasan® 114) was a gift from Sasol (Germany). Stannous chloride dihydrate was purchased from Rankem RFCL Limited (New Delhi, India). Solvents used for TLC and HPLC analyses were of HPLC grade (Spectrochem, India). All other chemicals were of analytical reagent grade and were purchased from S. D. Fine Chemicals, Mumbai, India.

3. Methods

3.1. Characterization of tacrolimus-loaded lipid nanoparticles (T-LN)

T-LN dispersion was formulated using hot melt emulsification combined with high pressure homogenization technique and developed to a gel for topical application as described in our previous report (Pople and Singh, 2010). The composition of T-LN is provided in Table 1. Gaussian unimodal size distribution analysis was performed using Beckmen N5 Submicron Particle Size Analyzer (Beckmen, Inc., USA) based on the principles of dynamic light scattering (DLS). The scattered light intensity was detected at scattering angle of 90° at 20 °C. T-LN dispersion was diluted with double distilled water so as to adjust the light scattering intensity between $5e+004$ and $1e+006$. Size distribution processor (SDP) analysis was performed from the scattered light intensity fluctuations to provide the best evaluation of polydispersed distribution. Results represent an average of three different measurements. Unimodal fingerprint

analysis of T-LN was performed to determine the presence of contaminants if any and reproducibility in particle size range.

3.2. In vivo dermatopharmcokinetic (DPK) studies in guinea pigs

Tape stripping approach was employed to investigate the penetration of tacrolimus in skin. Technique involved physical removal of surface cell layers of stratum corneum (SC) using an adhesive tape (Herkenne et al., 2006; Lindemann et al., 2003). Concentration of drug in the corneocytes attached to the adhesive strips was then correlated to the penetration of drug.

Fur on dorsal trunk of adult guinea pigs (weighing 350–400 g) was removed carefully 24 h before the commencement of study and areas of 2.25 cm^2 were marked using a template. The experimental procedure involved application of 150 mg (0.1% (w/w) of tacrolimus) of test formulation on the back of the animal in an occlusive manner. The study was planned in such a way so as to provide information on drug uptake, steady state levels and drug elimination from SC. The time intervals were selected based on initial pilot study. The study was performed for 48 h and SC samples were collected at 0, 3, 6, 12, 24, 30 and 48 h. After the predetermined exposure period, skin at the treated site was cleaned with sterile cotton swabs. SC sampling involved removal of SC layers by validated tape stripping procedure (Shah, 1998). The SC at the treatment area was progressively removed by repeated adhesive tape stripping after applying constant and uniform pressure on the strips (Micropore Tape, 3M, St. Paul, Minnesota) and then by pulling at an angle of 45° using clean forceps. First two strips were discarded due to the possibility of drug remaining on the skin surface and strips 3–14 were collected for analyses. Drug content in the SC harvested by tape strippings was determined by quantitative extraction using high performance liquid chromatography (HPLC) analysis method (Herkenne et al., 2007, 2008) with UV detection at 210 nm. Analysis was performed on a XDB C8 column (particle size 5 μm) employing a mobile phase consisting of trifluoro acetic acid (TFA) and acetonitrile in a gradient program mode with a flow rate of 1.0 ml/min. The method was validated as per ICH guidelines (Pople and Singh, data unpublished).

SC concentration *versus* time data for tacrolimus in individual guinea pigs was analyzed by non-compartmental pharmacokinetics (Gopala et al., 2002; Wolfsegger and Jaki, 2009). Maximum SC concentration ($C_{sc\text{-}max}$) and the time to reach $C_{sc\text{-}max}$ ($T_{sc\text{-}max}$) were measured directly from the concentration *versus* time profiles. Area under the curve ($AUC_0^{48\,h}$) was calculated using the linear trapezoidal rule. The comparative bioavailability of tacrolimus in T-LN was calculated with respect to the reference formulation.

3.3. In vivo gamma scintigraphy in albino rats to study the skin localization and biodistribution

Radiolabeling of the formulations was carried out with ^{99m}Tc by direct labeling method using stannous chloride as reducing agent (Reddy et al., 2004). Prior optimization of labeling parameters was performed as previously described (Arulsudar et al., 2003).

Healthy adult Wistar albino rats weighing 200–250 g were selected for the study. One day prior to dermal application, dorsal hair was removed carefully avoiding any abrasion to the skin. Rats were anesthetized with diethyl ether and radiolabeled test formulations were applied topically (0.1% (w/w) drug, 100 mg) in an occlusive manner to the circular skin area of 3.14 cm^2. After application the site was covered with a protective sheet and the animal body was wrapped with an elastic bandage to prevent removal of the sheet. Before topical application, the activity in the dose was measured and considered as the pre-syringe count for subsequent calculations.

Static whole body imaging of the rats was performed to obtain information of localized activity and biodisposition. Rats were placed prone on a gamma camera equipped with a LEGP collimator and images were acquired at periodic intervals of 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h on count mode (60 s). A constant circular region of interest (ROI) limiting application site skin area (hot spot) was drawn on images. Activity of ROIs was counted to analyze the scintigraphic results. Activity at the adjacent area was used as background count. Percentage residual activity in the skin (treatment area) and treatment area-to-whole body ratios were calculated.

At predetermined time points of 0.5, 1, 2, 3, 4, 8, 12 and 24 h, blood samples were drawn by retro-orbital venous plexus puncture and plasma was collected by centrifugation of whole blood (5000 × *g*, 15 min). The collected blood and plasma samples were quantified for radioactivity. Localization of radioactivity in the skin (application site) was confirmed by sacrificing the animals at 4, 8 and 24 h after the application of test formulations using overdose of ether inhalation. Radioactivity in the carcass was measured. Skin at the application site was excised and analyzed for radioactivity using well type γ-scintillation counter (IC 4702). Tissues of interest (liver, kidneys, spleen, heart, lungs and muscle at the application site) were collected immediately after sacrificing the animals and analyzed for radioactivity to assess the biodisposition of drug.

3.4. In vivo efficacy studies in murine model of AD

Eight- to ten-week-old female BALB/c mice, weighing 16–18 g, were purchased from ACTREC (Advanced Centre For Treatment Research and Education in Cancer, Navi Mumbai, India) and acclimatized to the in-house laboratory conditions for 7 days. AD was induced on the ears of mice by hapten induction method (Jin et al., 2009). Sensitization was performed by topical application of 100 μl of dinitrofluorobenzene (DNFB) solution (0.5%, w/v) prepared in acetone:olive oil in the ratio of 80:20. After 5 days of sensitization, the animals were challenged on both surfaces of the ears with 20 μl DNFB solution (0.2%, w/v) to induce the inflammation. Two days later, the mice were re-challenged again on both sides of the ears with 20 μl of DNFB solution (0.2%, w/v) to yield the extensive disease. In BALB/c mice, repeated exposure to the sensitizing agent induces production of inflammatory cytokines. Therefore the procedure was repeated after every 2 days for 2 weeks to induce Th2 response mimicking human AD (Petersen, 2006). The mice were randomly assigned to the treatment and control groups with each group containing 6 mice. Treatment was conducted with topical application of T-LN enriched gel (100 mg) once a day to both sides of the ears till the symptoms of the disease disappeared (for 6 days). Results were compared with the reference. The placebos of both formulations were also evaluated. Group without any disease induction served as negative control while the group with disease induction but no treatment served as positive control for the study.

AD response was monitored by measuring the extent of earflap thickness using digital micrometer gauge and grading the skin reactions. Grading of skin reactions was done to determine the total dermatitis score (OECD 404). The improvement index (IMI) for T-LN and reference was calculated with respect to the placebo in order to assess the enhanced efficacy. The results were also confirmed histologically.

3.5. Acute and repeated dose dermal toxicity studies

Acute and repeated dose dermal toxicity studies were performed as described earlier (Singh and Pople, 2011) in accordance with OECD Guidelines 402 and 410. Briefly, albino Wistar rats weighing between 200 and 300 g were randomized and assigned to the treatment and control groups (each group contained six

rats). Test formulations were applied uniformly over depilated skin (approximately 10% of the body surface area). A 100 times higher dose of human exposure, *i.e.* 10 mg/kg was selected for acute dermal toxicity study while usable dose of 0.1 mg/kg was selected for repeated dose dermal toxicity study. For acute dermal toxicity, exposure period comprised of 24 h and the observation period was of 14 days. For repeated dose dermal toxicity, the animals were treated for 6 h per day on a 6-day per week basis, for a total period of 28 days.

Animals were observed and individual records were maintained throughout the study period for any changes in vital physiological functions of respiratory, circulatory, autonomic and central nervous system and pharmacological signs such as tremors, convulsions, salivation, diarrhea, lethargy, sleep somatomotor activity and behavior pattern. At the conclusion of study period, blood samples were collected for hematology and biochemistry determinations. All the animals in each experimental group were humanely sacrificed using ether anesthesia. The animals were dissected and major organs namely liver, kidney, heart, lung and spleen were collected and weighed carefully in order to calculate organ to body weight ratios. The tissue samples were preserved in formalin (10% buffered neutral) and processed for histopathology by cutting sections of 5 μm thickness and staining with hematoxylin and eosin.

3.6. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) and subsequent *post hoc* Tukey multiple comparison test. Mean difference with p value less than 0.05 was considered statistically significant.

4. Results and discussion

4.1. Characterization of T-LN

Formulation was prepared using high-pressure homogenization technique and characterized for physicochemical parameters. T-LN dispersion was fluid in nature with translucent appearance (Fig. 1, inset). Particle size analysis of T-LN revealed equal sized fingerprints for three repetitions demonstrating reproducibility and reliability of data (Fig. 1A). This was further confirmed by unimodal distribution profiles (Fig. 1B). The difference between three repetitions was not statistically significant using ANOVA ($p > 0.05$). SDP analysis provided the best evaluation of polydispersity and the actual distribution of particle sizes. The nanoparticle size of T-LN was found to be in the range of 20–150 nm (Fig. 1C). The formulation had an average particle size of 75 nm (Pople and Singh, 2010).

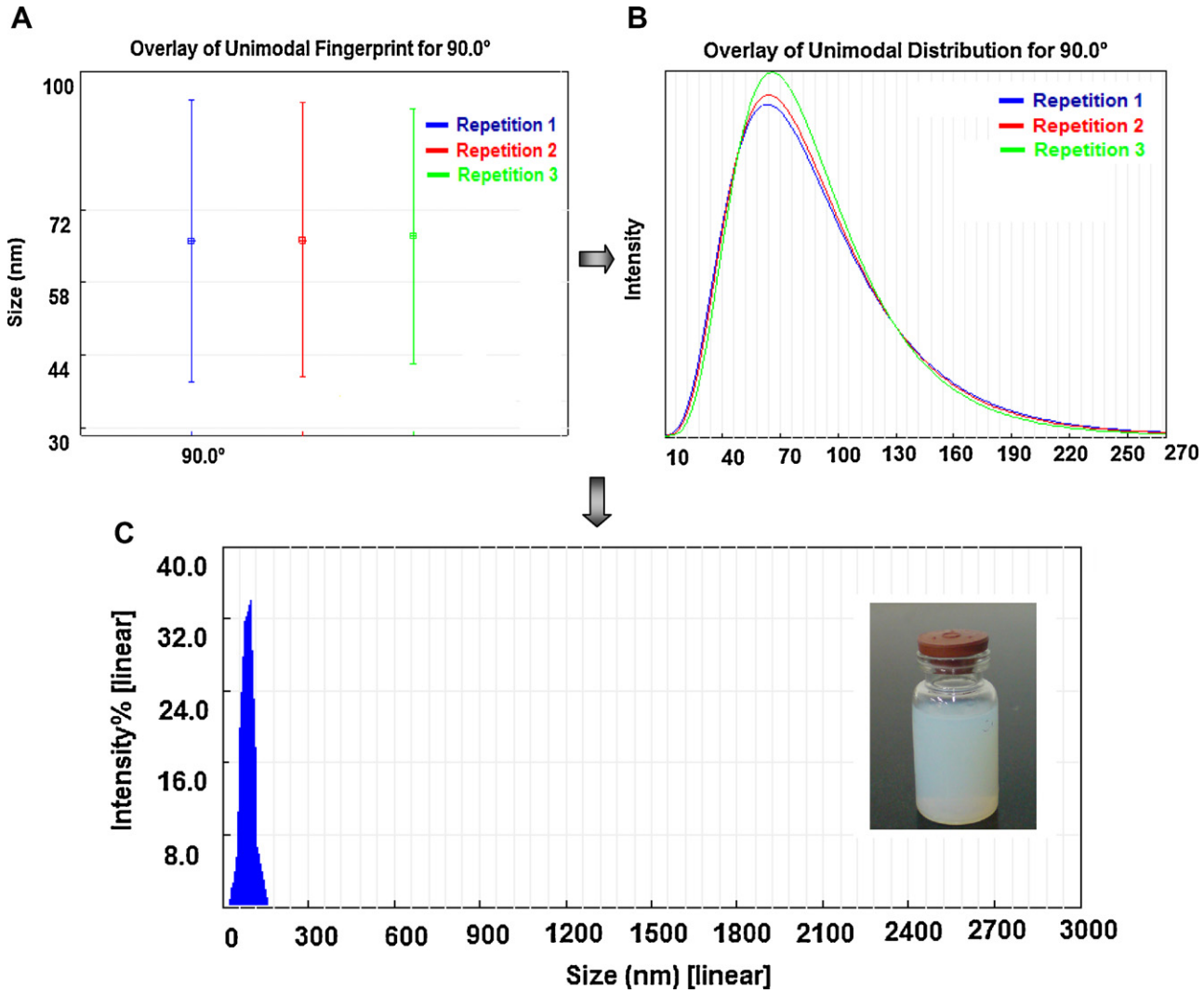

Fig. 1. Particle size analysis of T-LN. (A) Unimodal fingerprint and (B) Unimodal distribution for three repetitions of T-LN; (C) SDP histogram of particle size profile obtained by photon correlation spectroscopy (T-LN dispersion photograph in inset).

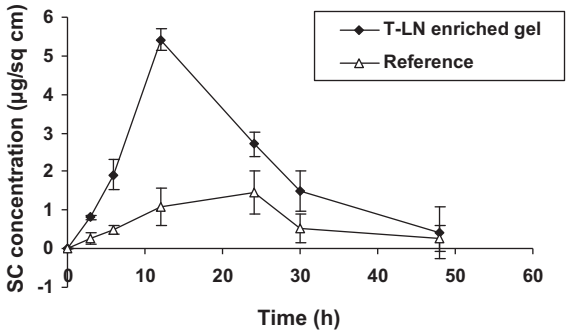

Fig. 2. Dermatopharmacokinetic data representing SC-concentration *vs.* time profiles of tacrolimus for T-LN enriched gel and reference.

T-LN dispersion revealed a narrow polydispersity index of 0.141. T-LN enriched gels designed for topical application were white in color, odorless with smooth texture.

4.2. In vivo DPK studies in guinea pigs

SC drug concentration–time curves of T-LN enriched gel and reference are depicted in Fig. 2. Tacrolimus SC concentrations were higher for T-LN at all time points examined. Comparison of several pharmacokinetic parameters revealed significantly higher values for T-LN than the reference (Table 2). One-way ANOVA revealed value of C_{max} to be significantly higher and T_{max} to be lower for T-LN enriched gel. Maximum concentration achieved with T-LN was 3.36 times higher as compared to reference demonstrating enhanced skin penetration and localization of drug as compared to the reference. These observations are consistent with our earlier reported data where permeation rate of tacrolimus through pig ear skin was almost 16–21 times higher for T-LN than the reference *in vitro* (Pople and Singh, 2010). In addition the values of $AUC_0{}^{48\,h}$ and $AUC_0{}^{\infty}$ were lower for reference formulation than T-LN enriched gel and the comparative bioavailability of T-LN was 3.02 times higher for T-LN. Thus, the higher SC drug concentrations in case of T-LN could be due to the higher drug release from the nano-enabled formulation.

Low skin penetration of drugs has been the main obstacle for the development of topical drug formulations for years. This is attributed to the significant barrier properties of SC, the outermost dead layer of skin (Bouwstra et al., 2003). It is important to note here that, even though the target site for tacrolimus to be effective is not the SC; it must still pass through the SC, to reach the deeper layers where site of action is located. In addition, it has been demonstrated that the SC functions as a reservoir for drugs, and concentration of drug in SC is a predictor of the amount of drug absorbed. Furthermore, several studies have indicated a positive correlation between the SC and deeper layer concentrations of the drug (Rougier et al., 1983, 1985). DPK studies clearly demonstrate that tacrolimus concentration is higher in SC with T-LN than the conventional reference. This in turn would result in increased bioavailability of tacrolimus at the effect compartment (epidermis and dermis) thus alleviating the symptoms of AD faster.

4.3. In vivo gamma scintigraphy in albino rats to study the skin localization and biodistribution

Gamma scintigraphy studies were performed to ascertain dermal targeting by focusing on skin uptake, localization and biodisposition of radiolabeled nanoparticles in rat skin to obtain detailed information at different times after topical application. Formulations were successfully radiolabeled using ^{99m}Tc with high labeling efficiency. Studies conducted to optimize the labeling parameters demonstrated that $SnCl_2$ concentration of 100 μg at pH 7.0 produced maximum labeling efficiency of 97.52% at 30 min incubation period. Determination of *in vitro* stability of ^{99m}Tc labeled complex demonstrated sufficient stability for up to 24 h.

Biodistribution data forms an integral part of targeted drug delivery and is necessary to show that the developed formulation is both effective and safe with reduced or no toxicity. After single dermal application of radiolabled formulations, the localization of radioactivity was much higher for T-LN than the reference at each selected time points throughout the study. Acquired gamma camera images showed specific localization of radioactivity in the skin (visible as hot spot in the scintigrams) (Fig. 3). Interestingly, from the total radioactivity detected in the whole carcass, major fraction remained localized in the skin without any ubiquitous spreading of radioactivity to other areas/organs of the body (Fig. 4).

Quantification of localized radioactivity demonstrated 1.5 times higher localization with T-LN (Fig. 5). These results indicate that more efficient dermal uptake of tacrolimus was achieved with T-LN further confirming our earlier results obtained with confocal laser scanning microscopy studies (Pople and Singh, 2010). Thus, encapsulation of tacrolimus in T-LN enhanced drug localization and confirmed its superior targeting ability to the skin. These results are in agreement with previous findings of *in vivo* DPK study (Section 4.2) demonstrating increased penetration of drug when applied as T-LN system. Radioactivity in blood and plasma was below the detection level during the entire study period. In addition, radioactivity counts in other major organs of the body namely liver, kidneys, spleen, heart and lungs were below the detection limit.

For treatment of AD, deeper skin layers were targeted and the objective was to maximize drug concentration at the site of action with, a minimal systemic uptake to avoid toxic effects. Cumulative amount of drug reaching the systemic circulation is associated with potential toxicity and undesirable side effects (Herkenne et al., 2008). Strikingly, T-LN delivered more amount of drug to the target site than the reference, and there was no dissipation of drug into the muscles at the site of application. This confirmed that the activity remained restricted at the application site avoiding non-specific distribution to other organs of the body thereby avoiding prospective drug related toxic side effects.

4.4. In vivo efficacy studies in murine model of AD

It is speculated that *in vivo* efficacy is dependent on the rate at which the drug penetrates the skin and becomes available at the site of action. To verify this, therapeutic efficacy of T-LN was evaluated *in vivo* using hapten-induced murine model of AD in BALB/c

Table 2
Dermatopharmacokinetic parameters (mean ± SD) for T-LN enriched gel and reference according to noncompartmental analysis method.

Formulation	T_{max} (h)	C_{max} (μg/cm²)	$AUC_0{}^{48\,h}$ (μg h/cm²)[a]	$AUC_0{}^{\infty}$ (μg h/cm²)[a]
T-LN gel	16	4.921	104.82 ± 18.26	120.11 ± 25.41
Reference	24	1.465	34.74 ± 15.78	76.25 ± 21.64

[a] Mean ± SD.

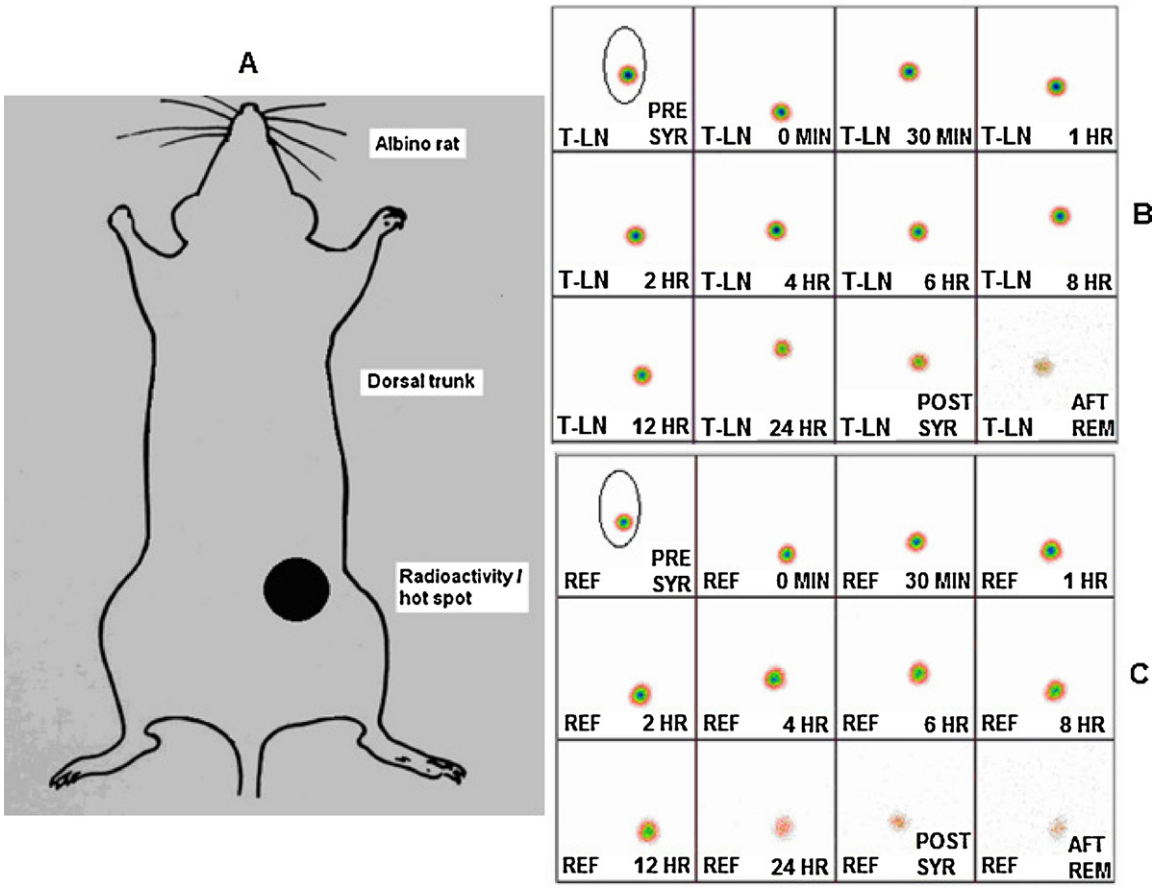

Fig. 3. Static whole body γ-scintigraphic images procured after periodic time intervals for 24 h after topical application (each scintigram is taken over a frame time of 60 s); (A) placement of radiolabeled formulation on the depilated dorsal trunk of albino rat; (B) T-LN enriched gel and (C) reference.

mice. Results using placebo gel and ointment vehicles were statistically identical. Therefore, only the results for placebo gel are shown (Fig. 6, C3 and C8). Reduction in inflammation for groups treated with T-LN (Fig. 6, C4 and C9) as well as reference (Fig. 6, C5 and C10) was significantly higher as compared with the placebo (Fig. 6, C3 and C8) and no-treatment groups (Fig. 6, C2 and C7). Interestingly, T-LN suppressed the inflammatory responses more efficiently than the reference (Fig. 6A and B). Topical treatment with T-LN enriched gel dramatically suppressed ear thickness, decreased total dermatitis score and the epidermal thickening immediately after drug application (day 1) while the reference required comparatively longer time to produce the same results. None of the placebo formulations alleviated the symptoms of AD . T-LN enriched gel demonstrated remarkably improved recovery in ear thickness and mean dermatitis score with respect to the reference during the study period. Recovery in mean dermatitis score was 1.29–2.0 fold higher for T-LN. Mean improvement indices for T-LN enriched gel and the reference were significantly higher as compared to placebo (Fig. 7). Strikingly, the improvement index of T-LN was 1.24 times higher for ear thickness data while 1.61 times higher for mean dermatitis scores as compared to the reference.

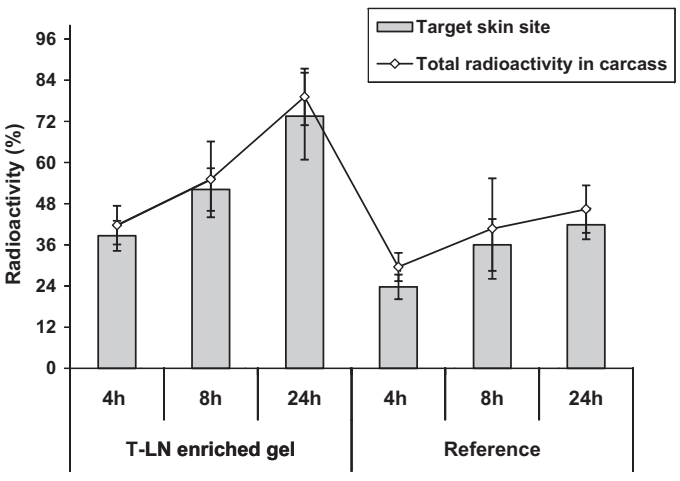

Fig. 4. Comparative assessment of radioactivity localized in the skin (application site) as compared to the whole body.

Previous studies on AD suggest that skin lesions are associated with Th2 type cytokine (IL-4, IL-5 and IL-13) expression and accumulation of large numbers of eosinophils (Leung and Barber, 2003), mast cells, and dendritic cells in the epidermis and the dermis of patients (Galli et al., 2005). Tacrolimus achieves immunosuppression by inhibiting the proliferation and activation of T helper cells by binding to the cellular receptor known as FK506-binding protein (FKBP). The tacrolimus-FKBP complex further binds to calcineurin, thereby preventing the dephosphorylation of the nuclear factor of activated T cells and blocking the cascade of cytokine gene transcription (Liu et al., 1991). It also inhibits the release of immune mediators from mast cells and basophils. Based on the fact that these T-cell related factors and other immune cells reside in the deeper skin, higher amount of tacrolimus in these layers would result in more efficient suppression of cytokines and other inflammatory mediators. This could ultimately lead to more efficient

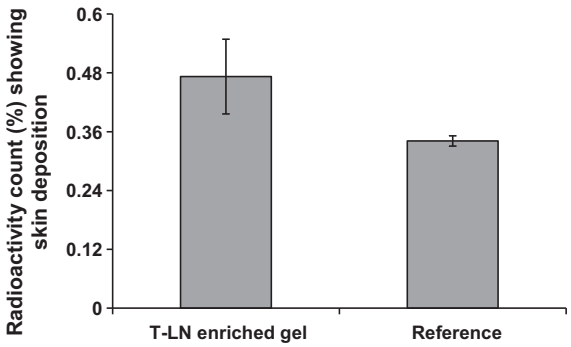

Fig. 5. Comparative skin deposition of radioactivity at the skin application area.

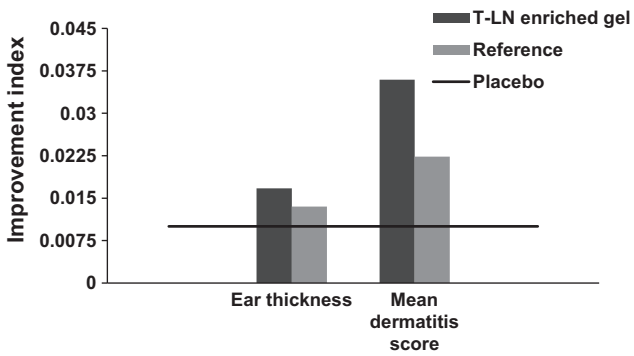

Fig. 7. Comparative profile of improvement index for T-LN enriched gel and reference with respect to placebo.

suppression of AD symptoms. The superior performance of T-LN is due to enhanced penetration, specific localization and increased bioavailability of tacrolimus at the site of application. Collectively, the above results indicate that T-LN is highly efficient in suppressing the inflammatory responses observed in hapten-induced murine model of AD.

4.4.1. *Acute and repeated dose dermal toxicity studies*

Our earlier data reported reduced skin irritation potential of T-LN enriched gel with Draize skin irritation study (Pople and Singh, 2010). Dermal toxicity studies provided detailed insight on any

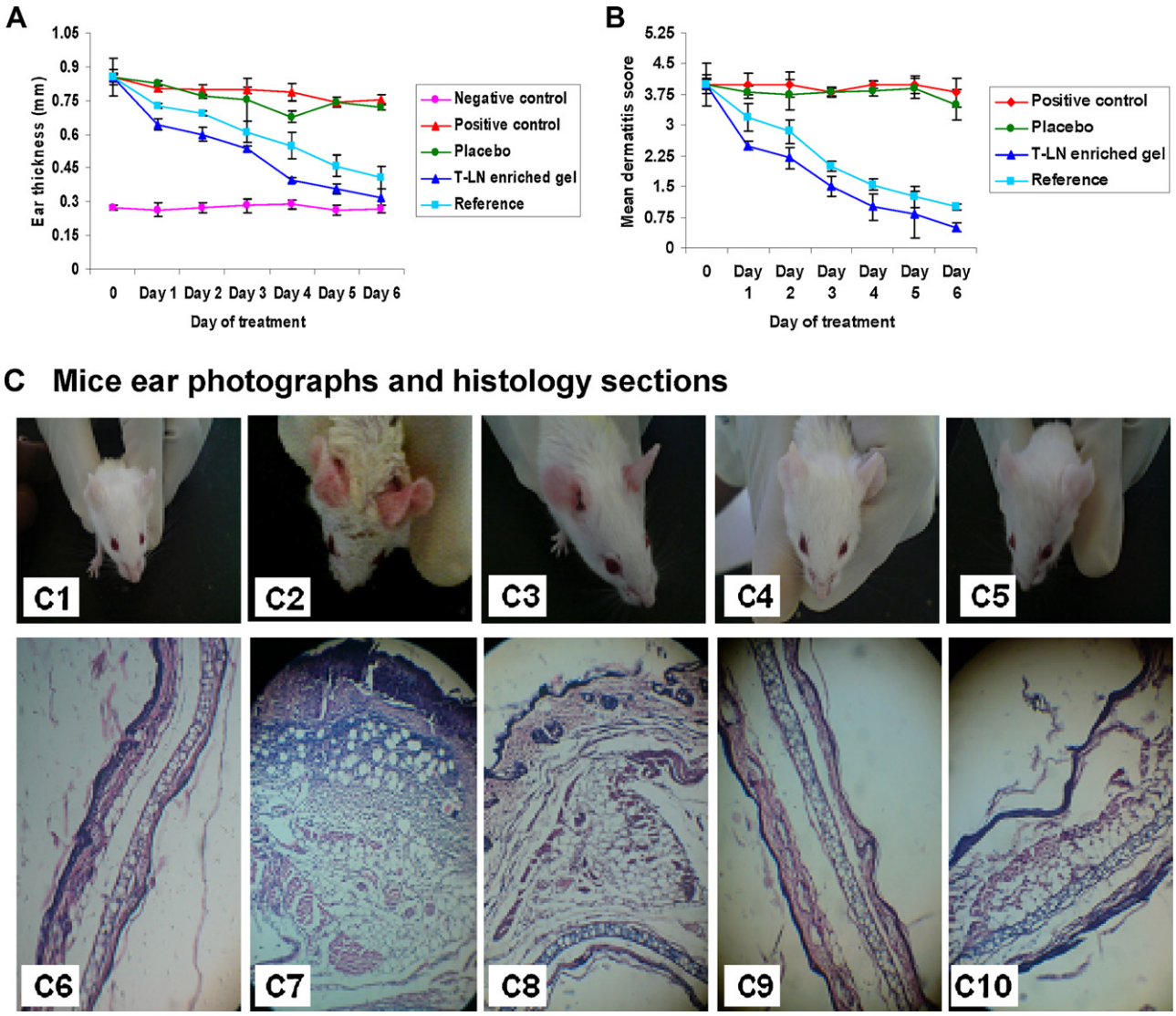

Fig. 6. Ear thickness scores (A) and mean dermatitis scores (B) in mice after treatment with test formulations. (C) The appearance of mice ears and histopathological observations; C1 and C6 show normal mice ear photographs and sections respectively. Note the marked inflammation (visible in photographs) as well as the thickening of the epidermis and dermal layers in positive control group (C2 and C7). C3 and C8 represent ears treated with placebo formulation. C4 and C9 show group treated with T-LN enriched gel while C5 and C10 represent group treated with reference. The results shown are representative of six mice in each group.

Table 3
Effect of repeated dermal application of tacrolimus on hematological parameters in 28-day dermal toxicity study.

Hematology parameters	Untreated control	Vehicle control	Group treated with T-LN enriched gel	Group treated with reference
Hb (g/dl)	14.7 ± 1.48	15.2 ± 0.09	14.5 ± 2.17	15.3 ± 0.72
PCV (%)	41.8 ± 0.74	43.3 ± 0.86	42.4 ± 1.28	44.4 ± 1.16
RBC (10^6/μl)	6.42 ± 1.10	6.08 ± 1.69	5.56 ± 1.20	5.78 ± 1.59
WBC (10^3/μl)	8.1 ± 1.46	9.7 ± 0.87	9.1 ± 0.81	8.8 ± 2.18
MCV (fL)	66.3 ± 1.73	63.6 ± 2.15	66.1 ± 0.98	67.1 ± 1.42
Platelets (10^3/μl)	9.53 ± 2.46	8.88 ± 1.12	8.94 ± 0.77	8.77 ± 1.14
Differential leucocytes count (DLC)				
N (%)	27.6 ± 1.54	28.3 ± 2.58	28.6 ± 2.73	28.2 ± 1.72
L (%)	75.6 ± 1.46	79.2 ± 2.59	69.7 ± 1.78	76.5 ± 1.45
M (%)	1.85 ± 0.55	1.98 ± 0.68	2.34 ± 0.56	1.66 ± 0.41
E (%)	0.52 ± 0.33	0.41 ± 0.22	1.28 ± 1.27	0.42 ± 0.56

Values are given as mean ± SD.

Table 4
Blood chemistry profiles of study groups on repeated dermal application in 28-day dermal toxicity study.

Blood chemistry parameters	Untreated control	Vehicle control	Group treated with T-LN enriched gel	Group treated with reference
S. CRE (mg/dl)	0.8 ± 1.72	0.5 ± 2.46	0.9 ± 2.29	0.7 ± 1.52
BUN (mg/dl)	13.4 ± 0.66	14.2 ± 1.72	13.1 ± 1.18	15.9 ± 1.46
BU (mg/dl)	16.3 ± 2.21	29.1 ± 0.68	37.3 ± 1.42	32.1 ± 0.58
UA (mg/dl)	4.1 ± 1.37	4.0 ± 2.16	3.1 ± 2.77	3.9 ± 1.24
S. Phos (mg/dl)	1.4 ± 0.45	1.8 ± 1.41	2.8 ± 0.46	2.0 ± 1.98
SGPT (IU/L)	26.3 ± 4.41	26.7 ± 2.58	23.4 ± 1.47	30.4 ± 2.29
SGOT (IU/L)	26.7 ± 3.57	30.9 ± 3.44	21.7 ± 2.46	27.7 ± 2.45
TP (g/dl)	6.5 ± 3.14	6.6 ± 2.66	6.6 ± 3.47	6.4 ± 2.46
ALB (g/dl)	4.2 ± 1.65	3.8 ± 2.45	4.0 ± 3.57	3.7 ± 1.02
GLO (g/dl)	2.0 ± 1.28	2.6 ± 1.17	2.7 ± 2.36	2.6 ± 1.35
CHO (mg/dl)	70.2 ± 4.77	71.4 ± 3.56	61.9 ± 4.48	68.4 ± 2.59
GLU (mg/dl)	108.8 ± 4.51	110.8 ± 10.3	107.2 ± 8.71	114.2 ± 6.21

Values are given as mean ± SD.

possible health hazards likely to arise from repeated exposures over a limited period of time. Acute as well as repeated application of test formulations did not reveal any abnormal changes in cage side observations. There was no mortality in any of the study groups. All study groups showed normal weight and exhibited no toxic effects during the study period. Clinical hematology and biochemistry parameters demonstrated no clinically significant differences ($p > 0.05$) between the untreated control and the study treatment groups (Tables 3 and 4). Kidney function tests, liver function tests, pancreas function test, nutritional status and lipids were not affected adversely by acute as well as repeated dermal exposure of tacrolimus to the treatment groups. Organ to body weight ratios were in accordance with the baseline data and did not reveal any significant changes as compared to the untreated control ($p > 0.05$) (Table 5).

Histopathology of all major organs such as liver, kidney, spleen, heart and lung did not reveal any distinct pathological alterations (Fig. 8). However, examination of application site skin area demonstrated dermal changes such as mild keratosis on repeated application of tacrolimus ointment. Repeated exposure of ointment showed slight collagenous mass infiltration in the subdermal layer, but no inflammatory reaction (Fig. 8). No such reaction was observed when ointment was applied to the skin for shorter periods. Surprisingly, group treated with T-LN enriched gel showed no evident toxicity even on repeated exposure, demonstrating significantly improved safety.

It is thus pertinent that the carrier in which the drug molecule is entrapped plays a critical role in influencing the penetration and transport of drug and hence the absolute amount of drug delivered to the site of action as well as safety of the drug. The entrapment of tacrolimus in lipid nanoparticles might have facilitated the drug–SC interaction due to smaller particle size as contrast to an ointment vehicle. Since the target site for tacrolimus is clearly beneath the rate limiting barrier (SC) of the skin (Lauerma and Maibach, 1994), the better the transport of drug through this barrier, the greater the therapeutic effect at the site of action where the dendritic inflammatory cells are present. Although, the precise mechanism by which T-LN might have penetrated into the deeper layers is not clear, the present work highlights the critical parameters influencing nanoparticle–skin interactions, which lead to improved *in vivo*

Table 5
Effect of repeated dermal application on organ to body weight ratio (%) of study groups in 28-day dermal toxicity study.

Organ	Untreated control	Vehicle control	Group treated with T-LN enriched gel	Group treated with reference
Liver	2.986 ± 2.68	3.953 ± 1.25	3.523 ± 2.11	3.357 ± 2.81
Kidney	0.652 ± 0.14	0.738 ± 0.61	0.645 ± 0.48	0.665 ± 0.36
Spleen	0.880 ± 0.31	0.509 ± 0.33	0.545 ± 0.32	0.711 ± 0.66
Heart	0.287 ± 0.082	0.309 ± 0.14	0.240 ± 0.18	0.277 ± 0.53
Lungs	0.509 ± 0.23	0.677 ± 0.42	0.369 ± 0.26	0.625 ± 0.22
Brain	0.755 ± 0.41	0.966 ± 0.21	0.551 ± 0.45	0.614 ± 0.06
Adrenal	0.0083 ± 0.06	0.017 ± 0.003	0.0079 ± 0.001	0.0049 ± 0.09
Ovaries and uterus	0.1038 ± 0.09	0.290 ± 0.18	0.385 ± 0.14	0.474 ± 0.18

Values are given as mean ± SD.

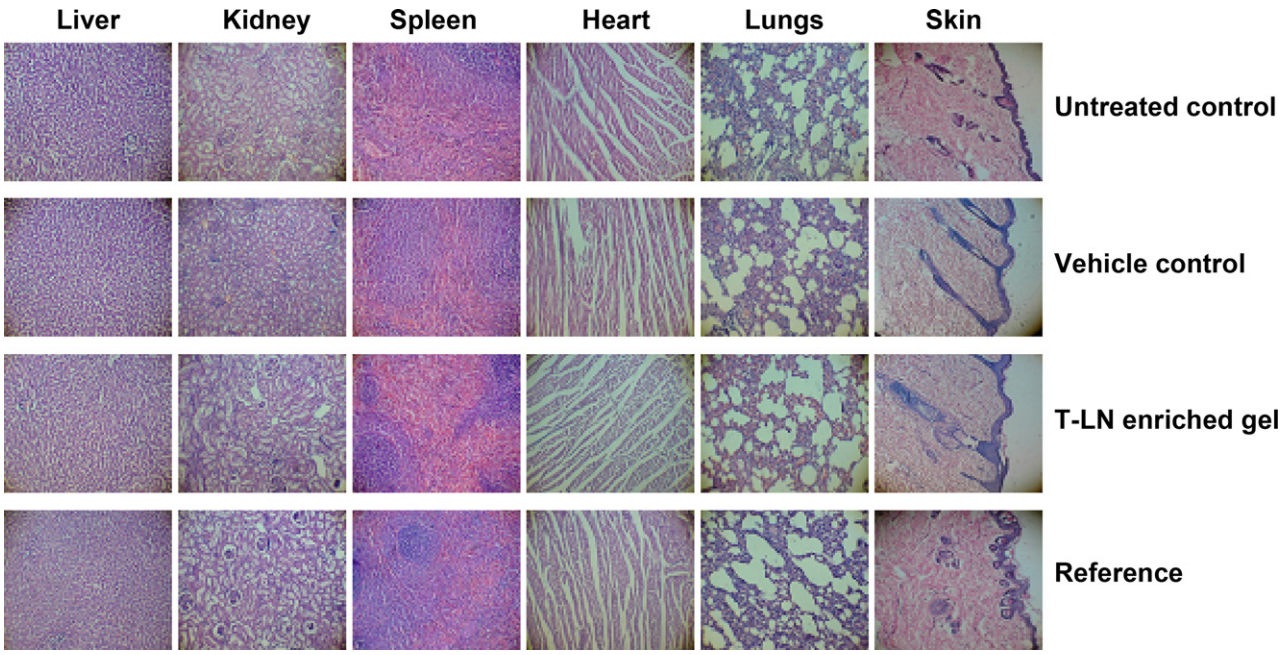

Fig. 8. Representative histopathological sections of various organs showing effect of repeated dermal application of tacrolimus in 28-day dermal toxicity study.

performance of tacrolimus in T-LN. The present study strongly demonstrates the formulation T-LN as a promising approach for topical delivery of tacrolimus in the treatment of AD.

5. Conclusion

In summary, T-LN demonstrated significant skin targeting potential for AD as compared to the reference. Topical DPK and γ-scintigraphy studies revealed strikingly improved drug penetration, localization and bioavailability at the site of action for T-LN without any general spreading and distribution to other organs of the body. Improved targeting resulted in enhanced therapeutic efficacy in murine model of AD without any toxic effects. Thus *in vivo* studies confirmed that T-LN would be more alluring and beneficial to the patient with better therapeutic efficacy, safety and without any greasiness inherent to ointment vehicles.

Conflict of interest

This research work is original and novel, and all authors have read and approved the text and consent to its publication. There is no conflict of interest.

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