



FK506-loaded PLGA nanoparticles improve long-term survival of a vascularized composite allograft in a murine model

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Background: The side effects of life-long administration of FK506 limit the clinical practice of vascularized composite allografts (VCAs). This study aimed to evaluate the feasibility of FK506-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles (FK506 NPs) for prolonging the long-term survival of VCAs and reducing the side effects of FK506.

Methods: PLGA nanoparticles loaded with FK506 were prepared by the solvent evaporation method. The characterization of FK506 NPs was evaluated by electron microscopy. To confirm the function and safety of FK506 NPs, these particles were administrated into rats by intraperitoneal injection. The survival time of the allograft, systemic concentration of FK506, anti-rejection activity, and side-effect of FK506 NPs were evaluated in a Brown Norway (BN)-to-Sprague Dawley (SD) epigastric VCA transplantation model.

Results: Compared with the nontreatment, PLGA control and FK506 groups, the median survival times (MST) of the FK506 NP groups were significantly prolonged. The FK506 NPs could maintain therapeutic drug concentration for 60 days. Moreover, cytokine concentrations, flow cytometry of regulatory T cells (Tregs) and histopathology of allografts revealed significantly prolonged immunosuppression by FK506 NPs. FK506 NPs also ameliorated FK506 nephrotoxicity.

Conclusions: FK506 NPs prolong the survival time of VCAs in a murine model with minimal nephrotoxicity, and provide a potential clinical strategy for ameliorating long-term side effects of immunosuppressive therapy.

Keywords: Flap transplantation; immune tolerance; poly (lactic-co-glycolic acid) nanoparticle (PLGA nanoparticle); FK506; vascularized composite allograft transplantation (VCA transplantation)

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Introduction

Transplantation of vascularized composite allografts (VCAs) is a promising option to restore complex anatomical and functional units with severe injury and defects that cannot be repaired by conventional reconstruction with autologous tissues or prostheses. Over the last two decades, several different VCA transplant, especially hand and face allotransplantations, have been successfully performed

with good functional and esthetic outcomes worldwide (1-6). However, due to the allogeneic nature of VCAs, severe immunological rejection occurs after transplant, especially with skin allografts. Over 87.8% of the upper extremity and 72.7% of the facial allotransplantation patients experienced at least one episode of acute rejection in the first post-transplant year (7,8). VCA transplant also leads to chronic rejection targeting mainly the allograft

vasculature, leading to graft vasculopathy and ischemic necrosis of the graft (9). As in solid organ transplantation, life-long immunosuppressive therapy to achieve long-term graft survival in VCAs is indispensable.

FK506 (tacrolimus), the first-line recommendation of calcineurin inhibitors, is commonly used in combination with mycophenolate mofetil and a steroid as a high-dose triple regimen for immunosuppressive maintenance in VCA transplantations, and this has been the mainstay in preventing and treating acute rejection since the 1980s (10-13). However, long-term systemic delivery of tacrolimus results in nephrotoxicity, hyperglycemia, hypertension, infection, and neoplasia (14-18). Additionally, higher rejection risks for VCA limits consideration of alternate immunosuppressive therapies. Therefore, future research in this field may primarily focus on the development of new FK506 formulations to achieve optimal immunosuppressive effects.

Biodegradable polymers, characterized by excellent bioavailability, biosafety, and controlled release, have been commonly used in a variety of nanocarrier drug delivery systems, such as microspheres, microcapsules, nanoparticles, pellets, implants, and films (19,20). Many studies have confirmed the capability of biodegradable polymers to prolong the survival of allografts without significant adverse effects (21-24). Tacrolimus-encapsulated glycolide-*co*-lactide-*co*-caprolactone polymer has been confirmed to prolong corneal allograft survival without adverse effects in rabbit eyes (25). In 2014, Xu *et al.* used degradable methoxy poly (ethylene glycol)—poly (lactide) (MPEG-PLA) to encapsulate FK506 and tested it on liver-transplanted Sprague-Dawley (SD) rats. Their results showed that the survival time of the rats treated with FK506-loaded MPEG-PLA nanoparticles was significantly prolonged compared to other groups, with better liver function (26). Encouraging data were also obtained in a Brown Norway-to-Lewis rat hindlimb transplantation model using tacrolimus-hydrogel which prolonged graft survival time to more than 100 days (27).

Poly lactic-*co*-glycolic acid (PLGA) is a widely used biodegradable material. The release kinetics of PLGA-encapsulated drugs are in conformity with the first-order kinetic equation (28). The high tacrolimus trough-level variability has been demonstrated as the detriment for long-term allograft survival in renal transplantations. However, the stable, sustained, and slow release of the encapsulated drugs indicates that PLGA may be a promising delivery system for VCAs (29). Therefore, in the present study, FK506-loaded nanoparticles were encapsulated in PLGA

to comprehensively examine the feasibility of using this method to prolong the survival time of allografts.

We present the following article in accordance with the ARRIVE reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-2425>).

Methods

Fabrication and characterization of FK506-loaded PLGA nanoparticles

Tacrolimus and PLGA (lactide:glycolide ratio 75:25, mol wt 66,000-107,000) were purchased from Sigma-Aldrich company (St. Louis, MO, USA). FK506 PLGA nanoparticles (FK506 NPs) were prepared by a solvent evaporation process described in detail previously (30-32) by the Pharmaceutical Technology Department of the University of Santiago de Compostela. A lactide/glycolide concentration ratio of 75:25 was used, and related studies confirmed the high encapsulation efficiency (33). The blank PLGA NPs were prepared by the same procedure without tacrolimus loading.

The morphology and size of the samples were observed and recorded using the Hitachi HT7700 electron microscope (Hitachi Ltd., Japan). Units of 6 mg FK506 were encapsulated with PLGA and lyophilized for 48 h, and the lyophilized solid nanoparticles were collected and stored at -20 °C. UV disinfection was required before administration.

The encapsulation and loading of tacrolimus into FK506 NPs were determined by analyzing the acetonitrile solution dissolved with weighted amount of FK506 NPs. Briefly, the solvents were diluted with acetonitrile, and then injected into a high-performance liquid chromatography (HPLC) with C₁₈ column (4.6 mm × 250 mm, 5 μm, Kromasil, Sweden) at 50 °C. The mobile phase was a mixture of double distilled water/acetonitrile = 70:30 (volume ratio) and the pH of water was adjusted to 4.0 by orthophosphoric acid, and the flow rate of the mobile phase was 0.7 mL/minute. Tacrolimus was detected at a wavelength of 210 nm. The drug-loading (%DL) and encapsulation (%EE) was calculated with the following equations: %DL = (weight of tacrolimus in FK506 NPs/weight of FK506 NPs) × 100 and %EE = (weight of tacrolimus in FK506 NPs/weight of tacrolimus used for FK506 NPs preparation) × 100.

Establishment of the animal model

Purified male SD and Brown-Norway (BN) rats weighing approximately 250–300 g were purchased from the

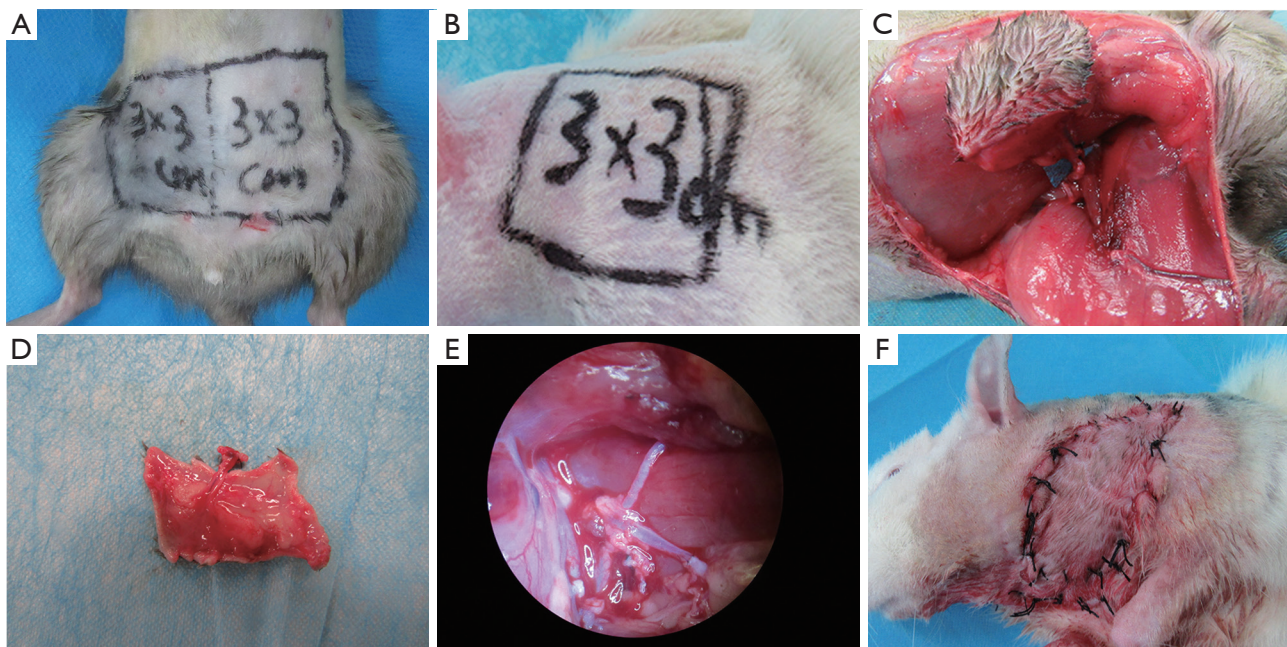


Figure 1 The operation procedure of VCA models. (A) The donor sites of BN rat, (B) the receptive site of SD rat, (C) the vascularized epigastric flap was harvested by cutting off the femoral artery/vein at the lower edge of the inguinal ligament, (D) the harvested allograft from BN rat, (E) the vascular anastomosis under operating microscope, (F) the established VCA model. VCA, vascularized composite allotransplantation; BN, Brown Norway; SD, Sprague Dawley.

Laboratory Animal Department of Central South University. The testing animals were kept in specific pathogen-free conditions. The animal studies were approved by the ethics committee of Xiangya Hospital of Central South University (No. 2020sydw0433) before implementation, in compliance with the national guidelines for the care and use of animals.

A BN allograft was transplanted to a SD recipient as described by Casal (34) and Ding (35). Under anesthesia by intraperitoneal injection of 3% (w/v) pentobarbitone, the vascularized epigastric flap was harvested by cutting off the femoral artery/vein at the lower edge of the inguinal ligament from the BN donor. The femoral artery/vein of the flap was anastomosed with the carotid artery and external jugular vein of the SD recipient at the level of a recently created carotid opening, using 11/0 nylon sutures and the flap was secured at the site where the skin had been removed. The wound was closed with interrupted sutures using 4/0 nylon sutures. The operation procedure is shown in *Figure 1*.

Grouping of animals

The allograft flap transplantation model was established using male BN rats as donors and SD rats as recipients.

Sixty-five SD rats underwent transplant, of which 17 were excluded from the study because flap failure within postoperative 3 days. Forty-eight SD rats were randomly by random number table divided into six equal groups (n=8 in each): no treatment (group I, control); empty PLGA nanoparticles (group II, NPs); single FK506 intraperitoneal (IP) injection (group III, S-FK506); single FK506 NPs IP injection (group IV, S-FK NPs); FK506 IP injection every 25 days (group V, M-FK506); FK506 NPs IP injection every 60 days (group VI, M-FK NPs). The first dose of immunosuppression in treatment groups was given at once after surgery (POD 0). Every administration contained 6 mg of FK506, and up to 4 doses were administered to multiple injection groups (group V, M-FK506 and group VI, M-FK NPs). The injection intervals in the M-FK506 group and the M-FK NPs group were based on the pilot trial in FK506 blood concentration.

Observation indicators

Graft survival time

The flap conditions, such as rejection and hair growth, of each group were observed daily postoperatively, and graft

rejection was evaluated macroscopically and graded as 0 (no rejection), 1 (erythema and edema), 2 (epidermolysis and exudation), and 3 (desquamation, necrosis, and mummification) (27). The endpoints were 60-day and 200-day allograft survival in single and multiple administration groups, respectively, or grade 3 rejection.

FK506 blood concentration

Ethylene diamine tetraacetic acid (EDTA) plasma samples were collected every 2 days after administration of FK506 in M-FK506 group and stored at -80°C until analysis. In M-FK NPs group, the samples were collected every 2 days in the first 10 days and then every 5 days until the next administration. Fifty microliters of EDTA plasma were analyzed using a PRO-Trac IITM Tacrolimus ELISA kit (DiaSorin Inc., Stillwater, MN, USA) according to the manufacturer's instructions.

Flow cytometry analysis of T regulatory cells (Tregs)

Peripheral blood mononuclear cells (PBMCs) were collected by Ficoll density gradient centrifugation on postoperative days (PODs) 3, 9, and 12 in control and NPs groups; 3, 7, 14, and 25 in S-FK506 group; and 3, 9, 30, and 65 in S-FK NPs group. Briefly, PBMCs were stained with anti-rat monoclonal antibodies (mAbs) anti-CD4-PE and anti-CD25-APC (eBioscience Inc., San Diego, California, USA) for 30 min, and then incubated with anti-FoxP3 mAbs (eBioscience) for 50 min after fixation and permeabilized by the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). After staining, PBMCs were analyzed by flow cytometry using a SORP LSRII flow cytometer and BD Diva Software (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJoTM software Version 10 (Tree Star Inc., Ashland, OR, USA).

Plasma cytokine levels

Levels of interleukins (IL)-2, IL-6, interferon (IFN)- γ , and transforming growth factor (TGF)- β in the plasma were evaluated at PODs 3, 7, 10, 20, 25, 30, 50, and 60 in control, S-FK506, and S-FK NPs groups. Briefly, 0.5–1.0 mL of tail vein blood was collected from each subject, and centrifuged at 3,000 rpm for 5 min to collect the supernatant, which was then frozen at -20°C . Ten microliters of supernatant were used to detect the changes in the concentrations of these cytokines using the ELISA kits (eBioscience).

Graft pathological examination

The rats were euthanized by intraperitoneal injection of

an overdose of pentobarbital. The graft tissue samples were collected on PODs 7 and 10 in control group and PODs 7, 15, and 30 in S-FK NPs group. The samples were preserved in 4% buffered formaldehyde and stained with hematoxylin and eosin, whereas paraffin sections were used for immunohistochemical (IHC) staining. The histological grading of skin rejection was performed according to the Banff classification (36).

Briefly, after a series of deparaffinization, rehydration, antigen retrieval, and blocking, sections were incubated with rabbit anti-mouse CD4 antibody (Abcam, Cambridge, UK) at 37°C for 60 min. Primary antibodies were then detected by incubation with goat anti-rat IgG polymer (PV-9004, ZSGB-BIO, Beijing), followed by DAB staining (ZLI-9017, ZSGB-BIO, Beijing). The IHC staining results were scored by positive cell rate (PR) and staining intensity. PR Score 0: $<10\%$; 1: $10\text{--}25\%$; 2: $25\text{--}50\%$; 3: $50\text{--}75\%$; 4: $>75\%$. Staining Score 1: weak staining; 2: moderate staining; and 3: strong staining. CD4⁺ T cell infiltration IHC score = PR \times staining. IHC score <1 , (-); 1.1–2.0 (+); 2.1–3.0 (++); 3.1–4.0 (+++); ≥ 4.0 (++++). (37,38).

Examination of liver and kidney functions

Creatinine, blood urea nitrogen, aspartate aminotransferase, and alanine aminotransferase levels were measured as indicators of side effects due to the use of tacrolimus. Plasma samples were taken on POD 100 from the non-operation group, M-FK506 and M-FK NPs group, and on POD 200 from M-FK NPs group. Creatinine, urea nitrogen, aspartate aminotransferase, and alanine aminotransferase (Jining Shiye, Shanghai) were assayed using a Hitachi 7020 automatic analyzer (Hitachi Ltd., Japan) according to the standard protocol in the enclosed pamphlets.

Statistical analysis

SPSS Version 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, USA) were used for statistical analysis and plotting graphs, respectively. The measurement data and results were reported as mean \pm standard deviation. The differences in survival time among various groups were compared and validated by the survival analysis log-rank test. Pairwise comparisons among multiple independent samples were performed using the one-way ANOVA-LSD test. The counting data were validated by chi-square (χ^2) test. $P < 0.05$ was considered as statistically significant.

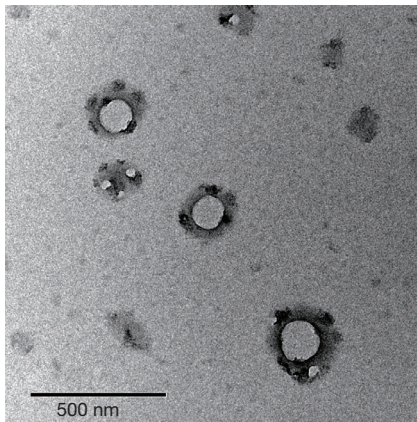


Figure 2 The establishment of FK506-loaded PLGA nanoparticles. The transmission electron microscope image of FK506 NPs (magnification, 20 k \times , the scale bar is 500 nm) and the average particle size of PLGA nanoparticles is 220 \pm 10 nm. PLGA, poly lactic-co-glycolic acid; NPs, nanoparticles.

Results

Preparation and characterization of FK506 NPs

Under the electron microscope, both FK506 NPs and NPs were similar in morphology and size, both having regular, smooth spheroidal cores, with an average particle size of 220 \pm 10 nm (Figure 2). The encapsulation efficiency and drug loading of FK506 NPs were found to be 85.73% \pm 1.73% and 7.42% \pm 0.42%.

FK506 NPs promote long-term survival in a rat VCA transplantation model

After transplantation, the mean survival times (MSTs) of allografts were 12.0 \pm 1.3, 11.6 \pm 1.1, 26.0 \pm 2.1, and more than 60 days in control, NPs, S-FK506 and S-FK NPs group, respectively (P=0.54, group control *vs.* NPs; P<0.05, group M-FK NPs *vs.* control, NPs and S-FK506; and P<0.05, group S-FK506 *vs.* control and NPs). The MSTs of groups M-FK506 and M-FK NPs were 112.4 \pm 4.2 days and more than 236 days (P<0.05, group M-FK506 *vs.* M-FK NPs). The MSTs were significantly prolonged in both single and multiple administration groups of FK506 NPs (Figure 3 and Table 1).

FK506 NPs maintain a longer effective blood drug concentration

Plasma levels of FK506 were compared between groups

M-FK506 and M-FK NPs. Direct administration of 6 mg of FK506 intraperitoneally after transplantation reached 207.2 \pm 17.6 ng/mL on POD 2 in group M-FK506. In group M-FK NPs, the FK506 concentration was notably lower (29.3 \pm 4.0 ng/mL) than in group M-FK506 (P<0.05). FK506 levels rapidly dropped to 103.1 \pm 13.5 ng/mL on day 6, and was 5.4 \pm 1.2 ng/mL on day 25 in group M-FK506. In contrast, FK506 levels decreased much more slowly in group M-FK NPs. On POD 25, the concentration of FK506 in group M-FK NPs (8.1 \pm 1.9 ng/mL) was higher than that in group M-FK506 (P=0.005), and then gradually decreased to 5.2 \pm 1.3 ng/mL on POD 60. After re-administration, systemic FK506 levels demonstrated consistent circulating drug level curves (Figure 4).

Proportion of Tregs during immunosuppression maintenance exceeds the inflammation period

On POD 3, the proportion of Tregs was relatively low in all groups and was maintained at 2.2% \pm 0.1% and 2.3% \pm 0.1% in groups control and NPs, respectively (P=0.255, group control *vs.* NPs). In Group S-FK506, The Treg proportion was 8.0% \pm 2.1% and 9.3% \pm 3.9% on POD 9 and 12, and then dropped to 2.8% \pm 0.8% on POD 25 when rejection occurred (P<0.05, POD 9, 12 *vs.* 3, 25 in group S-FK506). As for group S-FK NPs, the average proportion of Tregs was 2.1% \pm 0.8%, 8.0% \pm 1.4%, 9.3% \pm 2.1%, and 2.7% \pm 0.6% on PODs 3, 9, 30 and 65, respectively. The proportions of Tregs on POD 9 and 30 were significantly higher than on POD 3 and 65 in Group S-FK NPs (P<0.05, POD 9, 30 *vs.* 3, 65 in Group S-FK NPs), and there were no statistical differences between groups S-FK506 and S-FK NPs during the allograft survival period (P>0.05, POD 9, 12 in group S-FK506 *vs.* POD 9, 30 in group S-FK NPs; Figure 5 and Figure S1).

FK506 NPs reduce inflammatory cytokines

Inflammatory cytokines were induced in the rejection period and suppressed during immunosuppressive maintenance. The systemic levels of inflammatory cytokines in the control group were significantly higher than those in the FK506-treated groups on POD 10 (P<0.01). The levels of inflammatory cytokines in group S-FK NPs were significantly lower than those in group S-FK506 on POD 25 when rejection occurred in group S-FK506 but not in group S-FK NPs (P<0.01). The cytokine concentrations peaked on POD 60 when inflammation due to rejection was

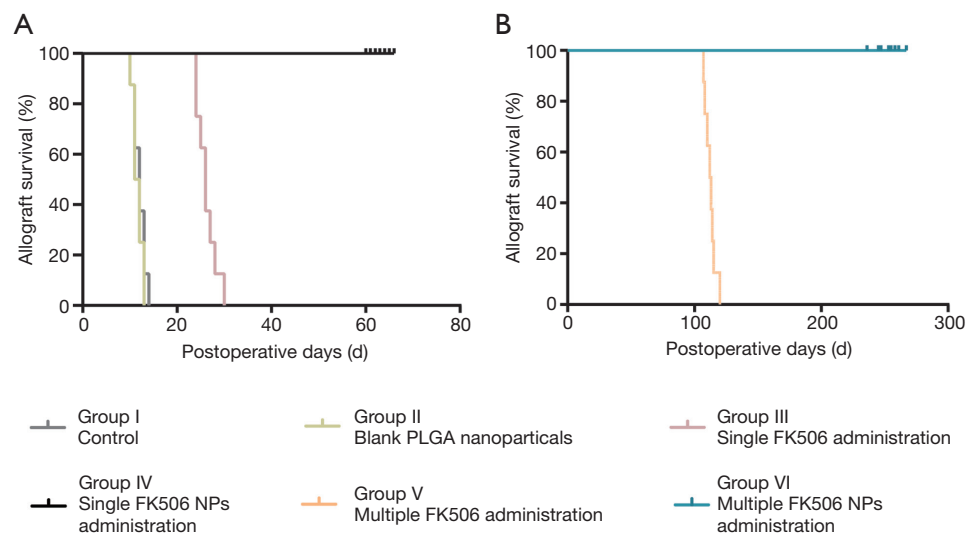


Figure 3 Survival time of each group. (A) The survival curve of single administration groups and (B) multiple administration groups. The mean survival times of FK506-loaded PLGA nanoparticles groups are significantly longer than other groups in both single and multiple administration groups. PLGA, poly lactic-co-glycolic acid.

Table 1 The mean survival time of each group.

Group	Treatment	n	MST (days)	P
I	No treatment	8	12.0±1.3	–
II	Blank PLGA nanoparticles	8	11.6±1.1	II vs. I: P=0.54
III	Single FK506 IP	8	26.0±2.1	III vs. I, II: P<0.05
IV	Single FK506 NPs IP	8	>60	IV vs. I, II, III: P<0.05
V	Multiple FK506 IP	8	112.4±4.2	
VI	Multiple FK506 NPs IP	8	>236	VI vs. V: P<0.05

MST, mean survival time; PLGA, poly lactic-co-glycolic acid; IP, intraperitoneal injection; NPs, nanoparticles.

augmented in group S-FK NPs (Figure 6).

FK506 NPs prolong immunosuppression

All recipients in group control showed acute rejection of the graft with edema formation and necrosis. Disruption of the skin architecture was observed on POD 7 and 10, accompanied by mononuclear cells and CD4⁺ T helper cell infiltration. The histopathological changes were notably more severe on POD 10 than on POD 7 in the control group; the Banff and IHC scores on POD 7 and 10 were Banff 2, IHC (++) and Banff 3, IHC (+++), respectively. In group S-FK NPs, there was no destruction of the skin tissue structure, and mononuclear cell and CD4⁺ T helper

cell infiltration were less severe on POD 7 than the control group. On POD 15, mononuclear cells, and CD4⁺ T helper cell infiltration vanished. Hair regrew on the skin graft on POD 30, no evident histopathological abnormalities were detected (Figure 7).

FK506 NPs are less nephrotoxic

The blood urea nitrogen and creatinine levels were notably higher in group M-FK506 (20.9±1.5 mmol/L and 53.0±7.6 μmol/L, respectively) than in the control and M-FK NPs groups (both POD 100 and 200) (P<0.01). The urea nitrogen in group M-FK NPs (10.5±1.0 and 10.7±1.1 mmol/L on POD 100 and 200, respectively) was also higher than

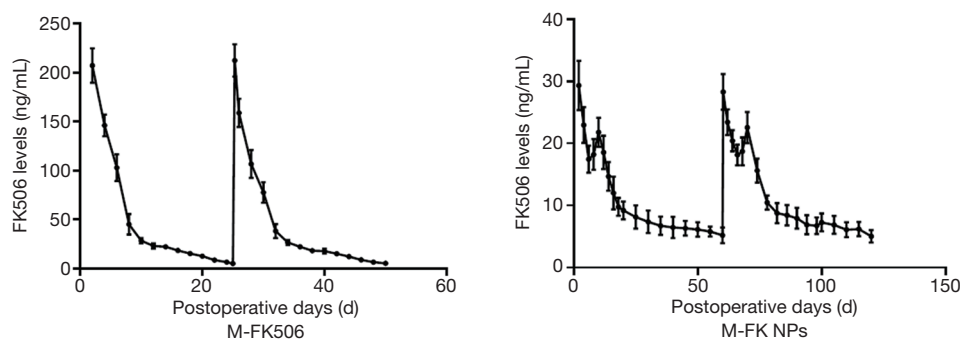


Figure 4 Systemic FK506 levels in group V (M-FK506) and group VI (M-FK NPs). (M-FK506) The initial burst release of tacrolimus IP peaks to 207.2 ± 17.6 ng/mL on POD 2, then rapidly fell to 5.4 ± 1.2 ng/mL on POD 25. (M-FK NPs) The concentration of FK506 decreases with time, and slightly increases during POD 6 to 10, and then gradually drop to 5.2 ± 1.3 ng/mL at POD 60. The release pattern of FK506 in group M-FK NPs is much flatter than group M-FK506. IP, intraperitoneal injection; POD, postoperative days.

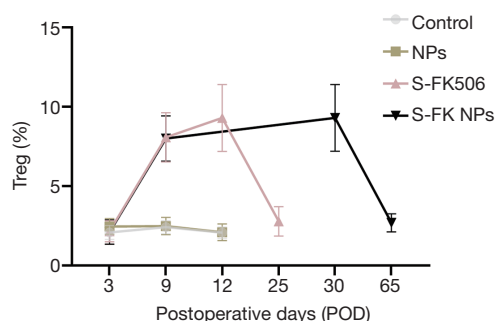


Figure 5 Treg subsets proportion in single administration groups. Flow cytometry of $CD4^+ CD25^+ FoxP3^+$ Tregs in peripheral blood in single administration groups. The Treg proportions of group I and II fluctuate at low levels. The Treg proportion during immunosuppressive period (POD 9, 12 in group III and POD 9, 30 in group IV) are significantly higher than inflammatory rejection period ($P < 0.05$, POD 9, 12 vs. 3, 25 in group III; POD 9, 30 vs. 3, 65 in group IV). CD, cluster of differentiation; FoxP3, forkhead box P3.

that in the control group (5.9 ± 1.1 mmol/L), but there was no difference in creatinine concentration between group M-FK NPs (22.9 ± 2.2 and 21.8 ± 3.2 μ mol/L on POD 100 and 200) and the control group (21.1 ± 2.0 μ mol/L) ($P = 0.11$, control group vs. group M-FK NPs). However, no statistically significant differences in aspartate aminotransferase and alanine aminotransferase concentrations were found among all groups (Figure 8).

Discussion

In clinical VCAs, long-term and regular administration of

immunosuppressants is imperative to avoid graft loss due to acute or chronic rejection. New strategies are required to improve patient adherence whilst reducing the side effects of long-term use of immunosuppressants.

Our data indicate that injection of FK506 NPs after transplantation prolongs allograft survival. In our study, 6 mg of tacrolimus encapsulated in PLGA as a one-time injection prevented allograft rejection for >60 days, which was also significantly longer than the MST of 26.0 ± 2.1 days in the 6 mg tacrolimus intraperitoneal injection group. Sustained, targeted, and controlled release of drugs contributes to allograft survival (39). Unlike the systemic administration of FK506, FK506 NPs exhibited a biphasic release pattern, characterized by an initial burst release followed by a slower release (31,40,41). The injection which contained tacrolimus to prevent FK506 NPs degradation before administration and the initial burst release of FK506 NPs caused the high systemic level in the immediate post-transplantation period. With the elimination of tacrolimus contained in the injection, the concentration gradient and the hydrolysis of the PLGA polymer accelerated the tacrolimus release in the POD 6–10 (40). In the following phase, tacrolimus is released progressively through the drug depleted layer. The therapeutic blood concentration of FK506 (5–15 ng/mL) (7,8,42) maintenance time of FK506 NPs (60 days) was notably longer than that of FK506 administration (25 days) ($P < 0.05$), which suggests that the extended release of tacrolimus at therapeutic systemic levels can prevent immune activation. This is in line with the sustained tacrolimus levels contributing to the inhibition of immune cell function by daily topical application of tacrolimus (40). The extended release of tacrolimus also

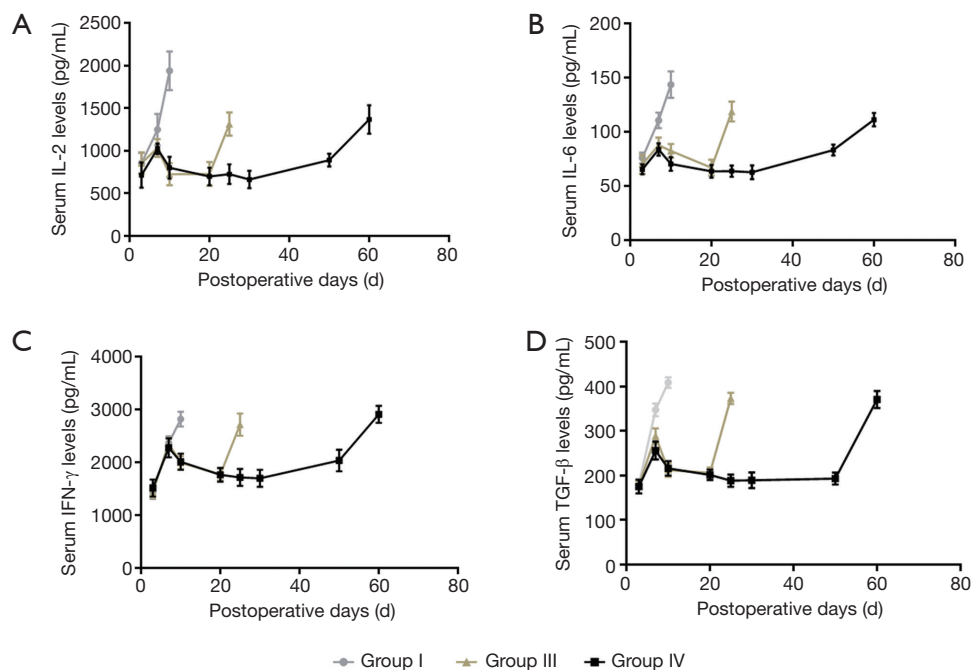


Figure 6 Systemic levels of pro-inflammatory cytokines in groups I, III and IV. The concentrations of (A) IL-2, (B) IL-6, (C) IFN- γ and (D) TGF- β in group I increase with time, and those concentration were significantly higher than FK506 treated groups III and IV on POD 10 ($P < 0.01$, group I vs. III; IV). The concentrations of cytokines rapidly increase after POD 20 in group III, and significantly higher than group IV on POD 25 ($P < 0.01$). The gradual increases of pro-inflammatory cytokines occur after POD 30, and peaked on POD 60 in group IV. IL, interleukin; IFN- γ , interferon γ ; TGF- β , transforming growth factor β ; POD, postoperative days.

means longer administration intervals which may improve medication adherence (43,44). In addition, the steady-state systemic levels of tacrolimus achieved by FK506 NPs prevents high systemic tacrolimus levels (as seen in tacrolimus IP injection), which may lead to toxicity (45-48). The steady-state systemic level of tacrolimus is also clinically significant for reducing the side effects of highly variable systemic tacrolimus levels in daily parenteral administration (29,49-51).

In our study, as in others, Treg upregulation was closely related to the prolongation of allograft survival (52,53). Lin *et al.* (54) achieved conventional CD4⁺ Tregs in the peripheral blood of tolerant VCA recipients in a mouse vascularized osteomyocutaneous transplantation model. In another model of heterotopic hind limb VCA in rats in which repetitive use of syngeneic adipose-derived mesenchymal stem cells promoted long-term allograft survival, there was sustained circulating Tregs levels observed (55). To some extent, the increase in the proportion of Treg represents the induction of immune tolerance (56,57). As for the inflammatory cytokines,

different cytokines have potential as markers for rejection (58,59). We observed increased levels of several cytokines (IL-2, IL-6, IFN- γ , and TGF- β) in the first week after operation and after the diminishment of tacrolimus, which is consistent with a previous study (27). Liu *et al.* (60) showed that FK506 loaded nanomicelles can remarkably reduce the amount of IL-2 and IL-17 to sustain the immunosuppressive effect. In a dynamic observation of serum cytokines in patients with hand transplantation for 5 months, it has been demonstrated that the low level of inflammatory cytokines (IL-2, IFN- γ , and TNF- α) is concordant with the survival of transplanted hands (61). As a result, FK506-loaded nanoparticles exhibited a strong immunosuppressive effect in the prevention of allograft rejection by sustaining higher Tregs proportion and prohibiting inflammatory cytokines.

Our data show that repeated intraperitoneal injection of tacrolimus-loaded PLGA nanoparticles sustained long-term graft survival with better toxicological outcomes than intraperitoneal injection of tacrolimus. Acute kidney injury was observed in group M-FK but not in group M-FK NPs.

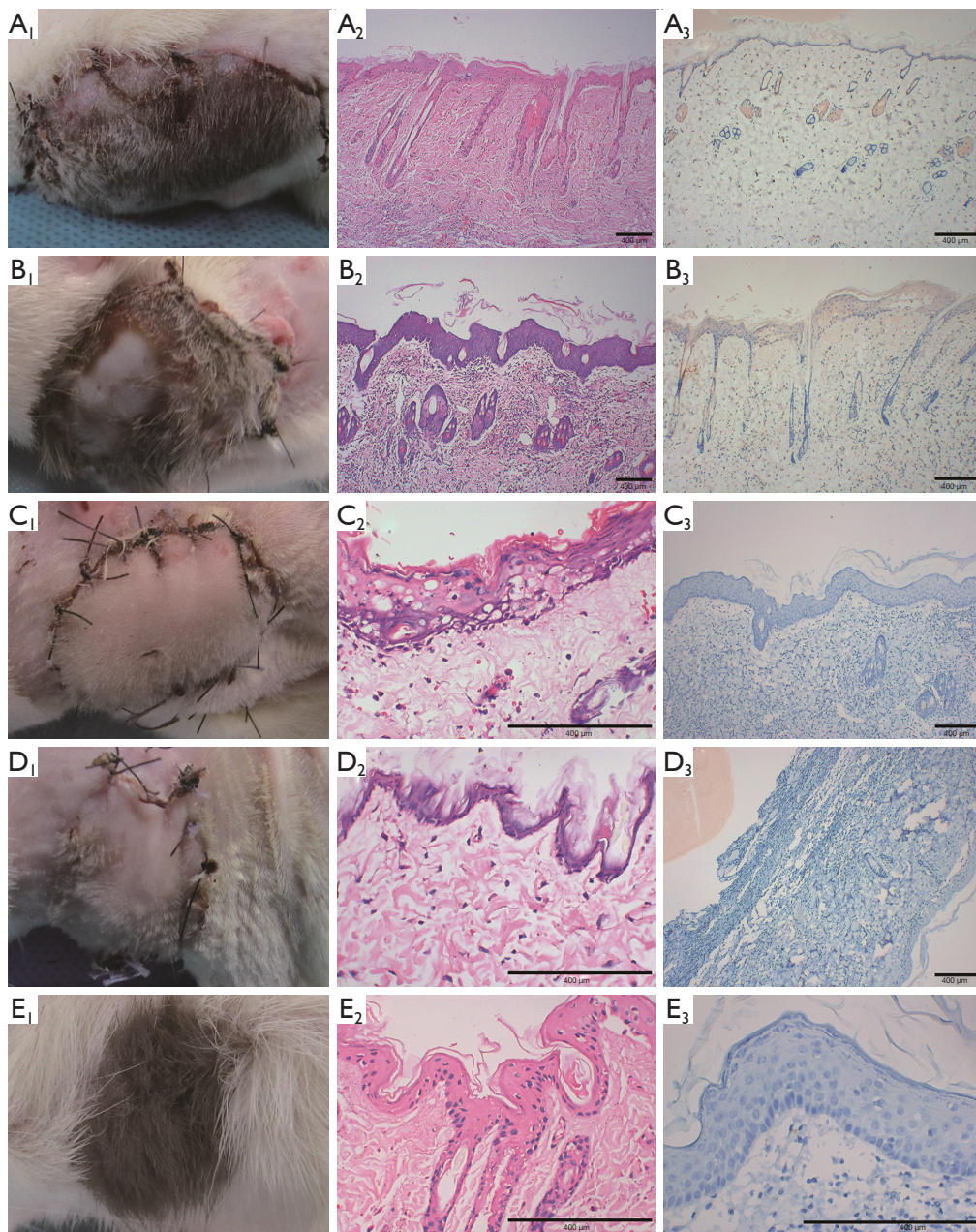


Figure 7 Histopathological features of vascular composite allograft in the control group (A,B) and group S-FK NPs (C-E). Representative macroscopic and photomicrographs of the histology (hematoxylin and eosin staining; CD4 immunohistochemical staining) images of allografts at POD 7 (A) and POD 10 (B) in the control group; and POD 7 (C), POD 15 (D) and POD 30 (E) in group S-FK NPs. Scale bar =400 μm . (A) A₁ showed an acute rejection at 7 days, A₂ showed a moderate amount of mononuclear cell infiltration in the dermis layer, the epidermis was intact. A₃ showed lightly staining in the lymphoid follicles and inter-lymphoid follicles. (B) B₁ showed deteriorated rejection on POD 10, the infiltration of mononuclear cell in the dermis layer increase, the epidermal cells were swollen (B₂), the staining in the lymphoid follicles and inter-lymphoid follicles deepened (B₃). The Banff and IHC scores on POD 7 and 10 were Banff 2, IHC (++) and Banff 3, IHC (+++), respectively. No signs of rejection were seen in group IV at day 7 (C₁), 15 (D₁) and 30 (E₁). The skin structure was normal on POD 7 in group IV, and a small amount of lymphocyte infiltration were observed, the Banff classification is grade 1 (C₂), and there is weakly positively staining in the lymphoid follicles and inter-lymphoid follicles (C₃). On POD 15 and 30 in group IV, no abnormalities detected macroscopically and microscopically (D₂, D₃; E₂, E₃). POD, postoperative days; IHC, immunohistochemical.

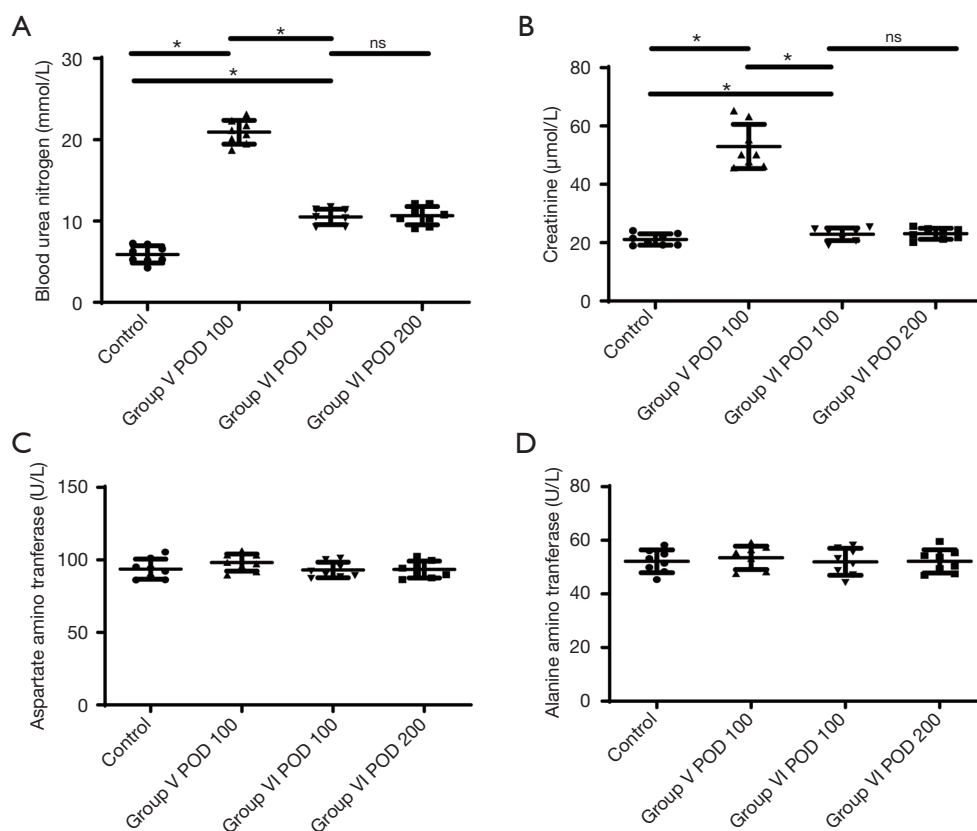


Figure 8 Indicator of kidney and liver functions. (A) Blood urea nitrogen level in group M-FK506 is significantly higher than the control group and group M-FK NPs on POD 100 and 200, and the concentrations of urea nitrogen in group M-FK NPs on POD 100 and 200 are also higher than the control group. (B) The amount of creatinine in group M-FK506 is significantly higher than the control group and group M-FK NPs on POD 100 and 200, while there is no statistical difference between the control group and group M-FK NPs. There is no statistical difference of aspartate aminotransferase and alanine aminotransferase concentrations among all groups (C,D) (ns, $P > 0.05$; *, $P < 0.05$). POD, postoperative days.

Acute nephrotoxicity has been attributed to systemic and renal vasoconstriction, leading to reduced renal blood flow and glomerular filtration rate, which is dose-related and reversible (62). The smaller fluctuations and lower systemic tacrolimus levels obtained by sustained release of FK506 NPs are beneficial to preserving kidney function.

As the one of the most commonly used biodegradable copolymer, PLGA was initially used as suture materials in humans (63,64). Due to its excellent biodegradability, biosafety and biocompatibility, PLGA has been approved by U.S. food and Drug Administration (FDA) as drug delivery carrier, tissue engineering scaffolds and resorbable prosthetic devices (65,66). Since the first approval of Lupron Depot based on PLGA microparticles in January 1989 by the U.S. FDA, only 20 drug products of injection

delivery based on PLGA formulations have been presented on the pharmaceutical market, of which 13 are micro-/nanoparticles, 3 are solid implant and 4 are in situ gel-forming implant (67,68). These clinical application of PLGA formulations have contributed to treatment of numerous diseases including cancer, diabetes or different immune disorders. On the other hand, the complicated design of a safe and effective PLGA formulation and the acidic micro-environment resulted from the hydrolysis of PLGA limit the clinical application (69,70). In addition, a more effective “on-demand” release of encapsulated molecules should be developed to enhance therapeutic efficacy. The negligible number approved by the U.S. FDA indicates the study and use of clinical PLGA formulations will continue to be a challenging field.

One of the major limitations of this study is intra-graft levels of tacrolimus were not tested. Several studies have highlighted the lack of a relationship between intracellular and blood concentration for tacrolimus (71), and the intra-graft level of immunosuppressive drugs is a more accurate marker of immunosuppression than trough levels. Therefore, pharmacokinetic studies analyzing tacrolimus intra-graft and intracellular distribution are needed. Additionally, our study is a rodent study only; further research on large animals such as pigs is required to evaluate the feasibility of FK506 NPs. IP injection is not likely to be applied in VCA recipients, indicating that the effect of different administration routes such as intragraft injection or intravenous injection on the systemic and intragraft distribution of FK506 needs to be investigated.

Conclusions

Drug administration through FK506 NPs prolonged the survival time of flap allografts with less nephrotoxicity in a rodent model. However, further research in large animals, such as pigs, is needed before transition to humans. The effects of long-term application of PLGA nanoparticles also need to be investigated.

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Footnote

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