Cyclosporine Nanomicelle Eye Drop: A Novel Medication for Corneal Graft Transplantation Treatment

Hongkui Zhang,* Ling Wang, and Longlu Zhang
Department of Ophthalmology, Teaching Hospital, Ningbo University School of Medicine; 247 Renmin Rd, Jiangbei District, Ningbo, Zhejiang 315020, China.
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Corneal transplantation has been used to treat severe eye disease for decades, but the therapeutic effect of the operation is highly compromised by immunological allograft rejection. To improve the success rate of corneal transplantation, we studied the protective effects of cyclosporine nanomicelle eye drops (CNED) on immune rejection after high-risk corneal transplantation and its underlying mechanisms. The therapeutic effects against immune rejection of both conventional cyclosporine eye drop (CCED) and CNED in different concentrations were assessed and compared using animal models of corneal transplantation. In addition, the expression of nuclear factor-κ-gene binding (NF-κB) as well as its target intracellular adhesion molecule 1 (ICAM-1) in the corneal samples obtained from recipients treated with either CCED or CNED was screened. The results showed that the CNED displayed significantly better effects at suppressing the immune response induced by corneal transplantation compared to CCED. CNED also significantly down-regulated the NF-κB and ICAM-1 expressions, indicating NF-κB might play an important role in the initiation of an immune response against the allograft. Our study demonstrates CNED may suppress the NF-κB pathway to attenuate the immune response, which highlights the possible therapeutic applications of cyclosporine nanomicelle eye drops in corneal transplantation.

Key words    cyclosporine; nuclear factor-κ-gene binding (NF-κB); corneal allograft transplantation; intracellular adhesion molecule 1 (ICAM-1)

Corneal transplantation is the most successful form of solid tissue grafting that serves as a crucial therapy in treating blindness clinically. Immunological allograft rejection is considered to be the leading cause of corneal graft failure. Though the 5-year survival rate of low-risk keratoplasty is approximately 90%, the survival rate of high-risk keratoplasty remains below 50% due to immune-mediated rejection even with maximal local immune suppression. The high-risk allograft host is characterized by vascularized and inflamed recipient beds, and the pre-existing corneal blood and lymphatic vessels in recipient beds were identified as the primary risk factors for immunological allograft rejection. Blood vessels circulates nutrients, oxygen and cells in blood vasculature while lymphatic vessels deliver antigenic materials and donor-derived antigen-presenting cells to the lymph nodes, subsequently inducing immune response against the corneal transplant.

Pharmacotherapy for corneal allograft rejection has considerably reduced the rate of graft failure. However, the therapeutic strategy based on corticosteroids remains unimproved over the past decades. Multiple side effects of corticosteroids have been observed including cataracts, glaucoma and opportunistic infections, and the EC50 of the drug varies in either the prevention or the treatment of allograft rejection. Considering the high failure rate of transplantation and the low efficiency of current treatment strategy, the development of alternative therapeutic regimens has become the first priority in corneal transplantation.

Cyclosporine is an essential medication used in the therapy of allogeneic hematopoietic stem cell transplantation. Research shows that cyclosporine could efficiently attenuate allograft transplantation rejection in both animal model and clinical trial. It has been implied that cyclosporine might suppress the expression of regulatory molecules related to the initiation of immune response. Intracellular adhesion molecule 1 (ICAM-1) was identified as one of the risk factors involved in the development of transplantation rejection, and the inhibitory effects of cyclosporine on ICAM-1 expression were also revealed. Though a growing body of evidences have revealed the therapeutic value of cyclosporine in the prevention and treatment of corneal transplantation rejection, the mechanism by which cyclosporine might suppress transplantation rejection and its effect on ICAM-1 expression remained elusive.

Moreover, the efficacy of cyclosporine treatment varies depending on dose and application methods. To improve the therapeutic effect of cyclosporine on allograft rejection prevention, we developed a soluble cyclosporine nanomicelle eye drop (CNED) with fewer side effects. The aim of the research is to evaluate the effect of the CNED on preventing corneal transplantation rejection compared to conventional cyclosporine eye drops (CCED) in a concentration based study. Furthermore, suppressive effects of cyclosporine on the expression of nuclear factor-κ-gene binding (NF-κB) and ICAM-1 were examined to illustrate how NF-κB might direct transplantation rejection by initiating the transcription of factors including ICAM-1 that may induce immune response. By understanding the role of NF-κB in transplantation rejection and its relation to cyclosporine treatment, we might be able to identify the mechanism by which cyclosporine may attenuate allograft transplantation rejection.

* To whom correspondence should be addressed. e-mail: hkzhang1234@163.com

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MATERIALS AND METHODS

Animals Fifty Sprague-Dawley (SD) male rats and 25 Wistar male rats, weighing 160–180 g, 8–10 weeks old, were purchased from Shanghai Banyao Company, China. The rats were housed for a week and the temperature and humidity of the animal house is 22–25°C and 50±5%, respectively. Fifty SD rats were used as recipients while the 25 Wistar rats were corneas donors. The recipients were randomly divided to 5 groups with 10 rats each: negative control without cyclosporine treatment (control), positive control with 2% conventional cyclosporine eye drop treatment (CCED, Allergan Pharmaceuticals), the 0.5%, 1%, and 2% cyclosporine nanomicelle eye drop treated groups (CNED), respectively. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Teaching Hospital. The IACUC committee members at Teaching Hospital approved this study. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. All of the donor and recipient mice were anesthetized by intraperitoneal injection of 3% pentobarbital sodium before any surgical procedures. Corneas of both eyes were obtained from the donors, and they were sacrificed by CO₂ asphyxia death after obtaining the corneas.

Cyclosporine Nanomicelle Eye Drops (CNED) Preparation A 0.5%, 1% and 2% CNED that contain 0.5%, 1% and 2% of cyclosporine (by weight) respectively were purchased from EPRUI Nanoparticles & Microspheres Co., Ltd. The CNED was manufactured based on the methods described by Song et al.13) with minor modifications. The material that was used to embed cyclosporine was polyurethane nanomicelle, which was also discussed as drug carrier by Song et al.

Induction of Corneal Neovascularisation Alkali injury was applied to induce corneal neovascularisation in the right eyes of the recipients with the methods described in previous study,14) though minor modification had been made to the methods. Briefly, circular filter discs with the diameter of 3 mm were immersed in 1 mol/L NaOH for 40 s, after which the discs were gently placed in the central area of the right eye upon the corneal surface of the recipients (fully anesthetized) for 40 s. The ocular surface was washed with 20 mL physiological saline for three times following the removal of the stimuli. The neovessels were expected to enter the mid-peripheral zone from the periphery after 14d, and only rats with neovessels in all four quadrants were eligible to be the recipients of orthotopic keratoplasty.

Corneal Transplantation Orthotropic corneal transplantation was conducted following the protocol provided by Gao et al.15) with minor changes. Both eyes of the donors were excised, and the central corneas of the donors were obtained by cutting carefully with a 3.5 mm trephine, the samples were stored in sodium hyaluronate for transplantation. The central corneas (with the diameter of 3 mm) of the recipients were excised to prepare the recipient graft bed. The donor button was placed on the recipient bed and secured in place with eight interrupted 10-0 nylon sutures, and the sutures’ end were cut short. 5-0 nylon was used to suture the eyelid to keep it closed for 24 h. The sutures were removed after 1 d while the transplant sutures remained untouched. Gentamicin and erythromycin eye ointment were applied after the surgery with the eyelid closed. The frequency of eye drop application was three times a day with one drop each. Images of the corneal allografts were recorded daily after the surgery for two weeks.

Clinical Evaluation Corneal graft rejection was evaluated based on the scoring system which involved the assessment of graft opacity, edema and vascularization, which was based on a scale of 0 to 4: 0=no graft opacity; 1=faint graft opacity with the iris texture clearly visible; 2=the iris texture is visible but opaque; 3=extensive opacity with the iris texture invisible, though the pupil can be seen; 4=total opacification. Graft edema was scored from 0 to 3: 0=absence of graft edema, the corneas were transparent and clear; 1=mild edema can be observed; 2=moderate edema; 3=significant edema can be observed with vesicles underneath the epithelium. Graft neovascularisation was assessed based on a scoring regimen from 0 to 4: 0=no neovessel observed; 1=neovessels reached the periphery of the graft; 2=neovessels extended to the intermediary zone of the graft; 3=neovessels reached the central area of the graft; 4=neovessels had fully occupied the graft area. Rejection index (RI) can be calculated by combining the scores of graft edema, opacity and neovascularisation, and RI≥6 was considered as the indicator of graft rejection.

Hematoxylin and Eosin (H&E) Staining To assess the efficacy of CNED, two rats were randomly chosen from each group after 14d of treatment, the rats were sacrificed and the eyes were excised, fixed in 10% formaldehyde. The corneal samples were embedded with paraffin, and sample slides with the thickness of 4 μm were fixed with 4% paraformaldehyde, stained by haematoxylin-eosin, observed under light microscopy.

Western Blot The corneas with transplantation of the recipients were excised after 14d, and the corneas homogenate (2mg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer was allowed for 2 h using semidry transfer cells after the completion of electrophoresis (120V, 2h). Polyvinylidenedifluoride (PCDF) membranes were incubated with blocking buffer (5% skim milk in PBST) for 30 min and then washed with phosphate buffered saline (PBS) for three times. Rabbit anti-NF-κB p65 antibody (Sigma, 1:100) or rabbit anti-ICAM-1 antibody (Sigma, 1:100) was used as primary antibody for the incubation of the membrane over night at 4°C. After three times wash with PBS, the membrane was incubated with goat anti rabbit immunoglobulin G (IgG) (Invitrogen, 1:500) for two hours at room temperature. Densitometric analysis of the film was performed with GS-710 imaging densitometer (Bio-Rad Laboratories, U.S.A.) in transmittance mode.

Immunohistochemistry The expression of NF-κB p65 and ICAM-1 were evaluated by immunohistochemistry. The rats were sacrificed after 14d of treatment, the eye globes were excised and fixed with 10% formaldehyde and embedded with paraffin. Slides with the thickness of 4 μm were obtained and incubated with primary antibodies (rabbit anti-NF-κB p65 antibody 1:100, rabbit anti-ICAM-1 antibody 1:100), 4°C overnight. After washed with PBS for 3 times, 5 min each, the samples were incubated with secondary antibody (1:500) for 2 h RT. Diaminobenzidine (DAB) kit was applied to show the colour, and the number of the cells that showed positive results within five views was counted under microscope (400×), the percentage of cells that were showing positive responds...
were calculated.

**Quantification of Interleukin-2 (IL-2) mRNA Level by Real-Time Polymerase Chain Reaction (PCR)** Corneal samples were obtained from the recipient and they were fractured in the presence of liquid nitrogen, total RNA were purified with Trizol reagent (Life Technologies, Gaithersburg, MD, U.S.A.). Twenty microliters water containing 1 µg of total RNA was added to 0.2 µg random primers purchased from Life Technology (Gaithersburg, MD, U.S.A.) and incubated for 15 min at 65°C. cDNA samples were prepared using Ready-to-go You-primer First-strand kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Real-time PCR were conducted for 2 min at 50°C, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control gene, primers and fluorogenic probes for GAPDH and IL-2 were purchased from TaqMan (CA, U.S.A.). Data were analysed with Sequencer Detector software.

**Statistical Analysis** All experiments were conducted in triplicate and repeated at least three times. The statistical differences between the experimental and control groups were analysed by one-way ANOVA. Values are presented as mean±standard error the mean (S.E.M.). Statistical significance is accepted at **p≤0.05** which represents significant differences.

**RESULTS**

**Allograft Rejection** Corneal graft rejection was first evaluated *in vivo* following cyclosporine treatment based on the scoring system, by assessing graft opacity, edema and vascularization (see Materials and Methods). Within 14 d following transplantation surgery, the samples in control displayed severe graft rejection. High level of graft edema and severe opacity were observed at early stage, and the symptoms deteriorated during two weeks of observation. After 14 d, severe edema and opacity can be observed in the corneas of control and significant transplantation rejection was observed in 9 of 10 recipients (Fig. 1a, Ctrl). At early stage, the corneas of the recipients in CCED group (treated with 2% conventional cyclosporine eye drop) showed certain degree of rejection. Moderate graft edema and opacity were found in the recipient eyes, and developed neovessels were observed after 14 d (Fig. 1a, CCED). Graft rejection rate was identified in the allograft transplantation in 5 of 10 recipients after 14 d in CCED group. Moderate edema appeared in the corneas of 0.5%, 1%, and 2% CNED groups with the iris invisible, and it can be observed that the neovessels reached the periphery of the corneas. Mild graft edema, neovascularisation and opacity were observed at the early stage of recovery in the allograft transplantation with CNED treatments (Fig. 1a, CNED). The transplantation rejection rate of 0.5%, 1%, and 2% CNED groups after two weeks of treatment was 5 of 10, 3 of 10 and 2 of 10 recipients, respectively.

The RI in these five groups was further quantitatively analysed (Fig. 1b). The rejection index in the experimental groups after 14 d of recovery. Data were presented as mean±S.E.M. *p<0.05 vs. control, **p<0.05 vs. CCED.

![](image)
was suppressed in CCED group, which displayed an average R1 of 4.92±0.51 (n=10). The mean RIs of 0.5%, 1% and 2% CNED groups were 4.83±0.48, 3.99±0.56 and 3.17±0.45 (all n=10), respectively. The results indicated the graft rejection was significantly suppressed by CNED treatments in a dose-dependent manner. Meanwhile, the RI of 2% CNED group is significantly lower than CCED group, suggesting CNED is more effective in inhibiting graft injection than CCED.

**Cornea Survival Time** Cornea survival time refers to the number of days the corneal survived after transplantation until rejection appeared. The average survival times (days) in the experimental groups (all n=10) are 6.8±0.54, 11.02±0.93, 11.05±0.98, 12.83±1.14, 14.8±1.35, respectively (Fig. 2). Significant difference of the survival time between the cyclosporine-treated groups and control can be observed (p<0.05). The results indicated that both conventional and nanomicelle cyclosporine could inhibit corneal transplantation rejection. The therapeutic effects, assessed by both the survival time and the average RI, of 0.5% CNED group was almost the same as that of the CCED, suggesting the nanomicelle significantly improved the efficacy of the drug. Moreover, the survival time was significantly increased in 2% CNED group than that of

![Fig. 3. H&E Staining Results of the Corneas 14 d after the Transplantation in the Experimental Groups](image)

![Fig. 4. NF-κB, p65 and ICAM-1 Expression in the Corneal Samples after Transplantation in Control, CCED, 0.5% CNED, 1% CNED and 2% CNED Groups](image)

(a, b) Percentage of NF-κB, p65 and ICAM-1 immunostained positive cells in the experimental groups. Inner fluorescence images represent for the NF-κB p65 and ICAM-1 positive cells, respectively. Scale bar=100 µm. (c) Representative images of NF-κB p65 and ICAM-1 proteins in the groups by Western blotting analysis. (d) Relative NF-κB p65 and ICAM-1 protein expression normalized to GAPDH in the groups. Data were presented as mean±S.E.M., *p<0.05 vs. Ctrl, †p<0.05 vs. CCED group.
CCED group ($p<0.05$), again indicating a dose-dependent treatment outcome of CNED.

**Pathological Examination** Furthermore, 14 d after transplantation, the cornea slides were obtained and stained by H&E (Fig. 3). The thickness of corneas in control was considerably increased, which indicated severe edema. Moreover, large amount of inflammatory cells as well as disordered layers were observed in the slide. Whereas in both CCED and 0.5% CNED groups, the thickness of the corneas increased only slightly, very few inflammatory cells were found in the samples, and the layers were generally ordered. Only mild edema were observed in the corneas in the 1% and 2% CNED groups, and the distribution of layers were quite ordered comparing to the corneas in other groups.

**NF-κB p65 and ICAM-1 Protein Expression** To further study the underlying mechanisms, immunochemistry and Western blot were employed to analyse the expressions of NF-κB p65 and ICAM-1 proteins after cyclosporine treatment. Within 5 randomly chosen field of views (400× magnified), the percentage of cells that exhibited positive response in terms of NF-κB p65 or ICAM-1 were counted (Figs. 4a, b). Significantly less NF-κB p65 and ICAM-1 positive cells were observed in both CCED and CNED treatment groups ($p<0.05$ vs. control). Moreover, the NF-κB p65 and ICAM-1 positive cells were significantly less in 1% and 2% CNED treatment groups than that in CCED treated group ($p<0.05$). Furthermore, Western blot analysis verified that NF-κB p65 and ICAM-1 expression was down-regulated in cyclosporine treated groups (Figs. 4c, d), as evidenced by lower level of NF-κB and ICAM-1 expression in the corneal samples of CCED and CNED groups. Also, 1% and 2% CNED treatment induced significant down-regulation of NF-κB p65 and ICAM-1 expression compared to CCED treatment ($p<0.05$), indicating expressions of NF-κB p65 and ICAM-1 were also inhibited by the treatment of CNED in a dose-dependent manner. Besides, to examine whether the CNED reduced the activity of T cells, the expression of IL-2 in these experimental groups was checked. The results show that CNED treatment could inhibit IL-2 mRNA expression compared to control (Fig. 5).

**DISCUSSION**

Cyclosporine eye drop has been used in the treatment of corneal allograft rejection since 1970. Current cyclosporine eye drops developed for clinical application were normally prepared with maize, castor or peanut oil, and the oil-based eye drops were likely to induce side effects including hyperaemia, blurred vision, pricking, burning sensation and pain in the eyes. It is also reported that 2% oil based eye drop would possibly lead to corneal epithelial lesions. To circumvent the drawbacks, we developed cyclosporine eye drops that were produced with nanophase materials. The drug was embedded within biodegradable nanomicelles that have high biocompatibility, which could significantly decrease the side effects. The water-soluble particles carried more cyclosporine per unit volume compared to the conventional oil-based cyclosporine solutions, and the drug was encapsulated thus well protected by the particles, assuring high drug availability. The nanomicelle cyclosporine is relatively mild to the eyes compared to the oil-based eye drops, which may also reduce the pain caused to the patients. Furthermore, the oil-free eye drop could lead to a reduced secretion of tears, which prolonged the retention time of cyclosporine in the ocular surface, consequently improving the efficiency of the medication. Also, no differences in the animal behaviours, including weight, body temperature can be found following CNED treatment (data not shown).

It has been reported that 0.5–2% cyclosporine had positive effects on the treatment of corneal transplantation rejection without inducing severe side effects, and 0.05% cyclosporine was also used in the treatment of dry eye disease. Our high-risk rat corneal transplantation model had suggested that 0.5%, 1% and 2% cyclosporine nanomicelle eye drop (CNED) could effectively inhibit transplantation rejection, and the inhibitory effects were augmented in a dose-dependent manner. The therapeutic effect of 0.5% cyclosporine nanomicelle eye drop could compete with that of 2% oil-based cyclosporine eye drop, while effect of 2% CNED has even exceeded our expectation for cyclosporine eye drops, as evidenced by significantly prolonged survival time and the reduced RI compared to those treated by 2% CCED. Here, we used another drug, corticosteroid, as a positive control in our experiments. Though the therapeutic effects of 2% CNED were not as magnificent as what corticosteroids treatment had achieved (Fig. 6), the combination of 2% CNED and corticosteroids treatments successfully elongated the survival time further and achieved the lowest RI. However, there were no significant differences in survival time and the protein expression (NF-κB p65 and ICAM-1) between corticosteroid and the combination of 2% CNED and corticosteroids treatment groups (Fig. 6). This may be due to two reasons. One is these three parameters (rejection index, survival time, NF-κB p65 and ICAM-1) are not 100% linearly correlated. That means there is no guarantee of rejection index will correlate exactly with the other two parameters, as shown in Figs. 6a–d. We think this is the main reason to explain the statistical difference between these three parameters. Besides, the statistical differences rely on the total numbers of the samples. For Fig. 6a, rejection index are calculated by the sum of the scores of graft edema, opacity and neovascularisation (see Materials and Methods). In this case, the numbers of the samples in Fig. 6a are three times larger than Fig. 6b. These findings are contradictory with the
research results of preliminary studies,\(^\text{18}\) which concluded 0.5% cyclosporine had no significant effect on the inhibition of corneal allograft transplantation rejection. Hence, it is highly likely that the nanophase material encapsulation contributed to the sustained release of cyclosporine, which could possibly prolong the reaction time of the medication, and consequently improving the efficiency of cyclosporine. Corneal graft transplantation rejection was a complex process,\(^\text{19}\) it was initiated by T cells and regulated by multiple factors including the activation of cytokines and intracellular adhesion molecules.\(^\text{20}\) Cyclosporine significantly inhibits the activation of T cells by binding to the cytosolic protein cyclophilin,\(^\text{21}\) and decreases the release of lymphokines, a major factor involved in the initiation of immune response.\(^\text{22}\) The cyclosporine–cyclophilin complex targets calcineurin, a phosphatase that dephosphorylates nuclear factor of activated T cells (NFAT).\(^\text{23}\) NFAT is identified as a nuclear transcription factor that is regulated by both cytoplasmic and nuclear Ca\(^{2+}\) signalling.\(^\text{24}\) The increase in cytoplasmic Ca\(^{2+}\) level leads to the translocation of dephosphorylated NFAT into the nucleus, and high nuclear Ca\(^{2+}\) concentration is required for normal function of NFAT.\(^\text{25}\) The inhibition of calcineurin by cyclosporine results in the suppression of NFAT translocation,\(^\text{26}\) which consequently represses lymphokine expression and interleukin release, leading to a reduced function of effector T-cells and insufficient immune response.\(^\text{27}\) Our results suggested that cyclosporine could attenuate the immune response caused by corneal allograft transplantation rejection. However, whether the process was related to NFAT requires further study. Numerous studies have successfully developed therapeutic regimens regarding to the prevention of allograft rejection based on the suppression of a single factor (i.e., intracellular adhesion molecule or cytokine).\(^\text{28}\) However, considering the corneal allograft transplantation rejection was resulted from the coordination of multiple factors, the suppression of a single factor might have limited inhibitory effects. To develop a more effective clinical strategy, we started from the regulating the expression of those factors. NF-κB is a protein that is ubiquitously expressed in nearly all cell types.\(^\text{29}\) Cyclosporine is able to interfere with IκB degradation which might diminish the transcriptional activity of classical NF-κB signalling.
Nishiyama et al. reported that the presence of cyclosporine could prevent NF-κB from migrating into the nucleus and instead retains it in the cytoplasm.\textsuperscript{30} They proposed that in the early phase of NF-κB activation, cyclosporine may inhibit NF-κB/RelA from translocating to the nucleus and binding to its target sequence in the IL-2 gene promoter region that are involved in human peripheral T cell proliferation. As one of the Ca:\textsuperscript{2+} sensitive transcription factors, NF-κB is associated with the expression of cytokine, and is involved in the regulation of immune response to infections.\textsuperscript{31} NF-κB dimmers are normally sequestered in the cytoplasm by IκB.\textsuperscript{32} By revealing their ankyrin repeat domains, IκB is capable of masking the nuclear localization signals (NLS) of NF-κB proteins, constraining NF-κB in the inactive state.\textsuperscript{33} During immune response, the activation of NF-κB is achieved partially by the signal-induced degradation of IκB proteins resulting from ubiquitination.\textsuperscript{34} The activated NF-κB translocates into the nucleus in response to Ca:\textsuperscript{2+} signals, and binds to the response element (RE) sequence to form DNA/NF-κB complex which recruits coactivators and RNA polymerases that facilitate the transcription of genes that direct both innate and adaptive immune response through T- or B-cell activation,\textsuperscript{35} including IL-1, IL-2, IL-6, IL-12, interferon-beta, tumour necrosis factor, intracellular adhesion molecule 1, vascular cell adhesion molecule 1 and chemotactic factors.\textsuperscript{36} Our results indicated that the expression level of NF-κB closely corresponded to the development of transplantation rejection. Also, CNED treatment reduced the expression of NF-κB p65 protein in the corneal transplantation samples and the suppressive effects were considerable compared to that of the corticosteroids treatment group. The combined treatment of corticosteroids and 2\% CNED downregulated their expression (Fig. 6), which in turn may supress the immune response. To test our hypothesis, we further assessed the expression level of ICAM-1, a protein whose expression is under the regulation of NF-κB and required for mature exosomes to prime naive T cells.\textsuperscript{37} Sun et al. reported that the induction of ICAM-1 was blocked by the inhibition of NF-κB both \textit{in vitro} and in intact isolated hearts, and the upregulation of NF-κB level improved the ICAM-1 expression as well.\textsuperscript{38} It has been proposed that NF-κB might regulate ICAM-1 expression by either working as the transcription factor or changing the interactions of two or more transcription factors that regulates ICAM-1 expression. The up-regulation of ICAM-1 in response to antigen independent events leads to the attachment of neutrophils, monocytes and lymphocytes to the vessel walls, which facilitates the transformation of circulating monocytes to macrophages that are responsible for phagocytosis.\textsuperscript{39} The cells are also involved in the process of leukocytes binding to major histocompatibility complex (MHC) class I and class II antigens on endothelial cells, which consequently results in antigen-dependent immunological allograft rejection.\textsuperscript{40} Therefore, ICAM-1 is crucial to the initiation of transplantation rejection. Here, we considered the expression of ICAM-1 as the indicator of the expression of immune response-inducing-factors, whose transcriptions were regulated by NF-κB. It is proposed that NF-κB could regulate ICAM-1 expression by associating with the upstream regulatory binding site. In this study, we found that the ICAM-1 was expressed at positions where the expression of NF-κB was also identified. Also, the expression of ICAM-1 corresponded to that of NF-κB. These results led to the conclusion that the expression of ICAM-1 was indeed regulated by NF-κB. Moreover, the expression of both ICAM-1 and NF-κB were inhibited by cyclosporine treatment and corticosteroid treatment. Moreover, the combined treatment of 2\% CNED and corticosteroids achieved the lowest expression level of ICAM-1, and the reduction of ICAM-1 expression level also corresponded to that of NF-κB. Hence, it is highly likely that the translocation and/or activation of NF-κB were inhibited by cyclosporine, resulting in the suppressed expression of ICAM-1, which eventually leads to the anti-inflammatory effects exerted by cyclosporine as observed in our current study.

**CONCLUSION**

In summary, our results showed that the CNED has significant effects on the prevention and attenuation of allograft transplantation rejection compared to CCED, as evidenced by the significantly decreased rejection index, prolonged survival time and less inflammatory cells. Furthermore, the anti-inflammatory effects of CNED might be achieved by suppressing the expression of NF-κB. Our study demonstrates the therapeutic potential of CNED for attenuating the immune response.

**Conflict of Interest**  The authors declare no conflict of interest.

**Supplementary Materials**  The online version of this article contains supplementary materials.

**REFERENCES**

10) Brooks DE, Plummer CE, Kallberg ME, Barrie KP, Ollivier FJ.


