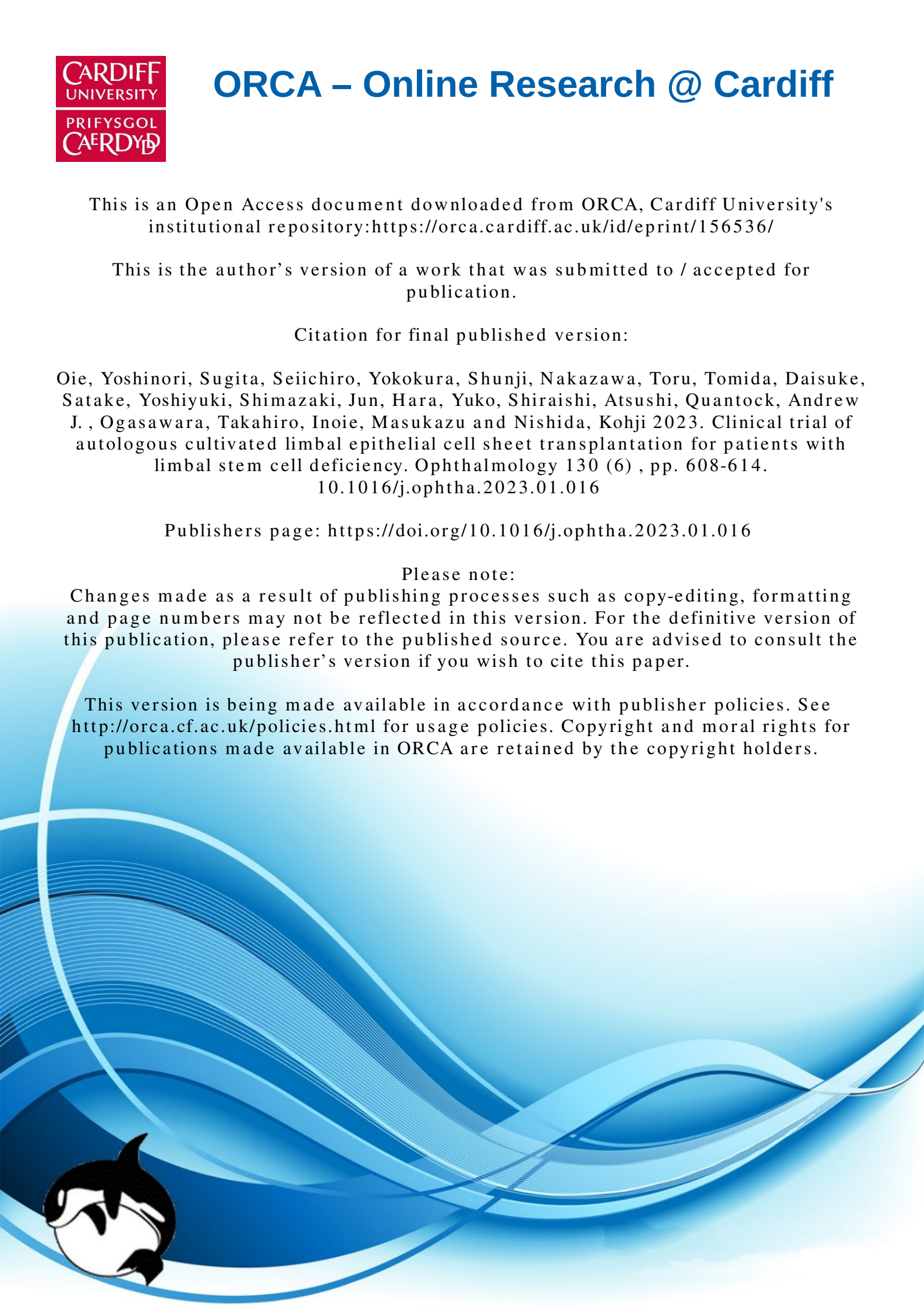

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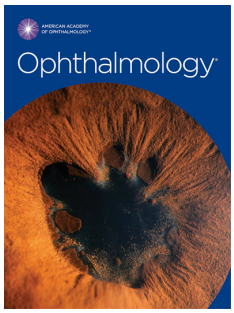

Clinical Trial of Autologous Cultivated Limbal Epithelial Cell Sheet Transplantation for Patients with Limbal Stem Cell Deficiency

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Running Head: Clinical Trial of Cultivated Limbal Epithelial Cell Sheet

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Abbreviations

CI: confidence interval; LSCD: Limbal stem cell deficiency; GCP: Good Clinical Practice; GMP: Good Manufacturing Practice; ETDRS: Early Treatment Diabetic Retinopathy Study; QOL: Quality of Life; MedDRA: Medical Dictionary for Regulatory Activities;

Keywords: Limbal stem cell deficiency, Cell sheet transplantation, Clinical trials, Tissue Engineering

ABSTRACT

Objective or Purpose: To confirm the efficacy and safety of Good Manufacturing Practice (GMP)-compliant autologous cultivated limbal epithelial cell sheets in government-controlled clinical trials that adhered to Good Clinical Practice stipulations for patients with unilateral limbal stem cell deficiency (LSCD).

Design: A prospective, multi-center, open-label, uncontrolled, single-arm clinical trial.

Subjects, Participants or Controls: Ten consecutive eyes of 10 patients with unilateral LSCD were followed for two years after surgery. Preoperative LSCD stage was IIB in four eyes and III in six eyes.

Methods: A limbal tissue biopsy was obtained from the healthy eye, after which limbal stem cells were dissociated and cultivated on temperature-responsive culture surfaces. All cell sheets were fabricated in a GMP-grade facility under established standard operating procedures. Cell sheets were evaluated using defined shipment criteria before transplantation, and only those that met the criteria were used. The cell sheet was transplanted onto each of the patients' diseased eye after removing the conjunctival scar tissue that covered the corneal surface. The severity of LSCD was determined according to a staging method agreed upon by global consensus, with eyes evaluated as being in stages IA–C representing successful corneal epithelial reconstruction. LSCD diagnosis and staging were determined by the trial's Eligibility Judgment Committee and Effect Assessment Committee using slit-lamp photographs including fluorescein staining. Both committees comprised two or three third-party cornea specialists, who were provided with information anonymously and randomly.

Main Outcome Measure: Corneal epithelial reconstruction rate was the primary endpoint.

Results: Corneal epithelial reconstruction was successful in six of 10 eyes (60%) one year postoperatively and was significantly higher than the 15% clinically significant efficacy rate achieved by allogeneic limbal transplantation. The reconstruction rate was 70% of eyes two years postoperatively. Additionally, improvements in visual acuity were noted in 50% and 60% of eyes at one and two years, respectively. No clinically significant transplantation-related adverse events were observed.

Conclusion: The efficacy and safety of cultivated limbal epithelial cell sheet transplantation were thus confirmed, and the cell sheet, named *Nepic*, is now approved as a Cellular and Tissue-Based Product in Japan.

INTRODUCTION

The cornea is a transparent tissue in the anterior part of the eye that transmits and focuses light into the eye. Its anterior surface is overlaid with a stratified, non-keratinized epithelium, which is constantly renewed by stem cells that reside in the basal epithelium of the limbus, a transitional zone between the cornea and adjacent conjunctiva.[1] Limbal epithelial stem cells are highly proliferative, express p63 and exhibit strong holoclone-forming capabilities.[2-4] However, if limbal stem cells are depleted because of congenital disease or injury, an opacified and vascularized conjunctival pannus will invade the cornea, severely disturbing vision.[5] This condition is known as limbal stem cell deficiency (LSCD). Although allogenic limbal transplantation has been used for treating LSCD, clinical outcomes are not always satisfactory because of postoperative complications, including infectious keratitis or immunologic rejection.[6,7]

Conversely, cultivated limbal stem cells have been successfully used for ocular surface reconstruction in patients with unilateral LSCD,[8,9] and it is expected that this surgical approach will become more widely used as the cell sheets become more readily available. Other clinical studies have described the use of *ex vivo* expanded limbal epithelial cells to treat LSCD,[10-12] but all these studies had limitations in that they either had a retrospective single-center study design or heterogeneity among transplanted cells because of the lack of evaluation under defined shipment criteria. More importantly, no clinical trial has been performed that adhered to Good Clinical Practice (GCP) stipulations within a defined clinical protocol and the use of strict quality control for cell sheets fabricated in a Good Manufacturing Practice (GMP)-grade facility. *Ex vivo* expanded autologous human limbal epithelial cell sheets containing stem cells have been approved as a medical product, *Holoclar*, by the European Medicines Agency. Here, we describe

what we understand to be the world's first government-controlled clinical trial, which has led to the approval of a Cellular and Tissue-Based Product that can be used to recover vision safely, effectively, and promptly.

MATERIAL AND METHODS

Study Design and Trial Oversight

We conducted a prospective, multi-center, open-label, uncontrolled, single-arm clinical trial to establish the efficacy and safety of graft surgery employing autologous cultivated corneal limbal epithelial cell sheets (the investigational product). The detailed clinical protocols are provided as Supplemental files 1 and 2. The trial had a postoperative follow-up period of one year, which was subsequently extended to two years with the same cohort. In the initial trial, patient monitoring comprised a pre-operative observation period and a follow-up period postoperatively (Supplemental Tables 1 to 3). The study protocol was approved by the Institutional Review Boards of each participating institute (Osaka University Hospital, Tohoku University Hospital, Ehime University Hospital, Sugita Eye Hospital and Tokyo Dental College General Hospital) and by the Ministry of Health, Labour and Welfare of Japan. Ten consecutive patients were enrolled between March 2015 and December 2016 after they provided written informed consent. The study was conducted according to tenets of the Declaration of Helsinki, sponsored by Japan Tissue Engineering Co., Ltd., and registered as UMIN000018969 and UMIN 000039994.

Patients and Endpoints

The inclusion criteria for the clinical trial were as follows: 1) Patients diagnosed with unilateral stage IIB or stage III LSCD,[5] with no improvement in conjunctivalization observed in the three months prior to screening for those with stage IIB disease; 2) presence of a healthy limbus in the uninjured eye, from which a limbal graft biopsy could be obtained for epithelial cell sheet fabrication without significant risk to that eye; 3) patients ≥20 years old at the time of providing informed consent. Detailed exclusion criteria are described in the clinical protocols (Supplemental files 1 and 2). Patients with treatment-resistant, severe decrease of tear or severe eyelid abnormality of the target eye were excluded. Use of the following drugs and therapies in the treated eye were prohibited during the study period to mitigate the risk that they may interfere with the evaluation of the study treatment: non-steroidal anti-inflammatory eye drops, drugs for glaucoma that might injure the epithelium (i.e. timolol maleate, betaxolol hydrochloride, and isopropyl unoprostone), artificial tear-containing preservatives, keratoplasty, curettage of the conjunctival epithelium from the cornea, amniotic membrane transplantation to the cornea and instillation of autologous serum. It is worth noting that this study was designed and initiated before the publication in 2019 of the global consensus on the definition, classification, diagnosis and staging of LSCD,[5] although we were involved with the working group for that report and much of our approach, reported here, aligns with the report's content, though not in its entirety.The trial's primary endpoint was the corneal epithelial reconstruction success rate (%) one and two years after the cell sheet transplantation. The severity of LSCD was determined according to a staging method agreed upon by global consensus,[5] with eyes evaluated as being in stages IA–C representing successful clinical outcomes. LSCD diagnosis and staging were initially conducted by on-site corneal specialists aided by slit-lamp examination including fluorescein staining and were finally determined by the trial's Eligibility Judgment Committee

and Effect Assessment Committee using slit-lamp photographs including fluorescein staining. Both committees comprised two or three third-party cornea specialists, who were provided with information anonymously and randomly. Two-sided 95% confidence intervals (CI) for the primary endpoint were calculated using the Clopper–Pearson method. Success rates one year postoperatively were determined using an exact binomial test at a two-sided alpha level of 5% to test the null hypothesis that the success rate would be 15%. (Supplemental File 1). The secondary endpoints for efficacy were as follows: 1) LSCD stage after transplantation of the investigational product; 2) subjective symptoms; 3) corrected visual acuity using a decimal visual acuity chart and an Early Treatment Diabetic Retinopathy Study (ETDRS) chart; 4) QOL, as evaluated by the 25-item National Eye Institute Visual Function Questionnaire; 5) severity of corneal opacity,[13] severity of corneal neovascularization,[13] and severity of symblepharon.[13] We also evaluated whether additional treatment to improve visual acuity was indicated one year postoperatively and whether further treatment contributed to the restoration of the corneal surface at the two-year time point in patients who received additional treatment. Visual acuity is presented as the logarithm of the minimum angle of resolution values, and improvements of two or more lines were regarded as significant. An improvement of one or more grades for corneal opacification, neovascularization, and symblepharon was considered significant. The occurrence of superficial punctate keratopathy or corneal epithelial defects for up to one year was assessed as safety criteria. Adverse events and malfunctions of the investigational device (i.e., cell sheet) were recorded and converted to standard terms using the Medical Dictionary for Regulatory Activities (MedDRA) /J Ver21.0.

Cell Sheet Fabrication and Quality Control

For each patient, a limbal tissue biopsy, approximately 3 mm^2 in size, was obtained from the healthy eye, after which limbal stem cells were dissociated and cultivated on temperature-responsive culture surfaces as previously reported[14,15] (Figure 1). Virus-validated, lethally irradiated 3T3-J2 cells from an established working cell bank were used as feeder cells. All cell sheets were fabricated in a GMP-grade facility managed by Japan Tissue Engineering Co. Ltd, Gamagori, Japan, under established standard operating procedures, guided and recorded under a process management system. Cell sheets were evaluated using defined shipment criteria before transplantation, and only those that met the criteria were used. The cell sheets were transported from the GMP-grade facility to the transplantation sites in a specialized container.[16]

Transplantation and Postoperative Care

A single expanded corneal epithelial cell sheet was transplanted onto each of the patients' eyes following procedures described in detail elsewhere.[14,15] Briefly, superficial conjunctival scar tissue that covered the corneal surface was surgically removed to expose bare corneal stroma to a distance of 3 mm outside the limbus. The cell sheet, lifted from its temperature-responsive culture dish, was then grafted directly onto the corneal stroma. A therapeutic soft contact lens was placed on the eye to protect the ocular surface. Postoperative local medication included topical antibiotics (0.5% cefmenoxime) and corticosteroids (0.1% betamethasone) as eye drops four times a day, along with betamethasone and fradiomycin ointment once a day. Betamethasone eye drops were switched to 0.1% fluorometholone eye drops 3–6 months after surgery, depending on the level of inflammation seen. Systemic steroids including 125 mg of methylprednisolone were administered on the day of surgery, followed by 2 mg of

betamethasone for two days and 1 mg of betamethasone for one month with tapering. Patients with severe dry eyes self-administered artificial tears.

Statistical Analysis Plans

The statistical analyses are described in Supplemental files 3 and 4.

RESULTS

Characteristics of the Patients

Twelve eyes of 12 patients matched all the inclusion and exclusion criteria as judged by the cornea specialists on site. However, one of these patients was deemed ineligible by the Eligibility Judgement Committee based on the inclusion criteria related to LSCD staging and adverse events that occurred in another patient before surgery. Therefore, the trial was discontinued for these two patients. Four of the remaining 10 eyes of 10 patients were treated at Sugita Eye Hospital and two each at Osaka University Hospital, Tohoku University Hospital and Tokyo Dental College Ichikawa General Hospital. The mean age of the patients was 51.1 ± 22.7 years at the time of enrollment (median age 47 years; range 20–83 years), and the cohort comprised seven men and three women. The cause of LSCD was chemical burn in six patients (alkali in five and acid in one), with the other four being either idiopathic or caused by mucous membrane pemphigoid (MMP), vernal keratoconjunctivitis, or long-term contact lens wear. Although, generally, some patients with MMP, vernal keratoconjunctivitis, and long-term contact lens wear are bilateral, the patients included in this trial were unilateral and had a healthy limbus in the uninjured eye from which a limbal graft biopsy could be obtained. The average

pre-operative ETDRS visual acuity was 1.65 ± 0.70 logMAR, and the LSCD stage was IIB in four eyes and III in six eyes (Table 4). The cultivated limbal epithelial cell sheets, which were generated for each patient as an autologous graft using cells taken from their healthy contralateral eye, all met defined shipment criteria (Supplemental Table 5). One eye of one patient with corneal stromal scarring underwent an anterior lamellar keratoplasty to recover vision 83 weeks after limbal epithelial cell sheet transplantation.

Endpoints for Efficacy and Safety

The corneal epithelium was successfully reconstructed; that is, the primary efficacy endpoint of the trial was reached one year postoperatively in six of 10 eyes (60%, 95% CI 26.2–87.8%) and was significantly higher than the 15% clinically significant efficacy rate defined in clinical protocol (Supplemental file1 and 2) (P=0.0028, Binominal test). Two years postoperatively, successful epithelial reconstruction was achieved in seven of 10 eyes (70%, 95% CI 14.7–94.7%). Representative cases are shown in Figure 2, and all LSCD staging is presented in Table4. Although corneal transparency was well maintained postoperatively in patients including C-1 and C-2, surgical outcomes in patient B-3 with MMP and A-3 who was idiopathic were complicated by severe early postoperative inflammation, and conjunctival invasion into the central cornea with neovascularization was observed.Of four eyes with a pre-operative stage IIB, three presented with stage IA at almost all postoperative visits. Of the six eyes with a pre-operative stage III grading, three exhibited stage IIB at one and two years postoperatively, with the other three consistently at stage IA.

The postoperative changes and grading of subjective symptoms are shown in Table 6 and Supplemental Tables 7–12. Overall, these changes were not substantive regarding ocular pain,

foreign body sensation, lacrimation, photophobia or dryness, which likely reflects that the pre-operative gradings were not high. Nevertheless, discomfort improved at one and two years postoperatively in six (60%) and four (40%) patients. As shown in Table 6 and Supplemental Tables 13 and 14, decimal visual acuity improved in six patients (60%) at one year and in five patients (50%) at two years postoperatively. Similarly, ETDRS visual acuity improved in five (50%) and six (60%) patients at postoperative years one and two, respectively. Quality of Life (QOL) scores, shown in Supplemental Table 15, improved in eight (80%) patients in postoperative years one and two. Corneal opacification also improved in eight treated eyes (80%) at postoperative years one and two, with corneal neovascularization improved in six (60%) and four (40%) eyes at these time points (Table 6 and Supplemental Tables 16 and 17). There was no significant change in symblepharon (Table 6 and Supplemental Table 18).

Regarding safety endpoints, superficial punctate keratitis was observed in three eyes (30%) pre-operatively and six eyes (60%) postoperatively. A corneal epithelial defect was present in two eyes (20%) pre-operatively and five eyes (50%) postoperatively. In general, the adverse events (Supplemental Tables 19 to 22) were not serious, and those that occurred after the limbal biopsy were expected events and readily managed. Likewise, adverse events after cell sheet transplantation were clinically insignificant and successfully managed by appropriate therapeutic intervention. We did not encounter any serious adverse events defined by clinical protocols (Supplemental files 1 and 2).

DISCUSSION

We conducted the world's first prospective, multi-center, government-controlled pivotal clinical trial of cultivated limbal epithelial cell sheets transplanted to the eyes of patients with

LSCD. Although similar clinical studies have been conducted,[10-12] no clinical trial has been performed that adhered to the GCP stipulations within a defined clinical protocol and the use of strict quality control for cell sheets fabricated in a GMP-grade facility. Our data, including the trial results reported here, were reviewed by the Ministry of Health, Labour and Welfare in Japan. The cultivated autologous limbal epithelial cell sheet was approved as a Cellular and Tissue-based product, named *Nepic,* in Japan. This is a model case of successful translational research that achieved approval as a novel product using stem cells.

The primary endpoints were evaluated objectively by third-party cornea specialists using anonymously and randomly provided clinical photographs without any accompanying information. Additionally, LSCD staging was objectively judged using fluorescein staining as recommended by a global consensus.[5] As a primary efficacy endpoint, the corneal epithelium was successfully reconstructed in 60% and 70% of eyes at postoperative years one and two, respectively. Visual acuity significantly improved in 50–60% of the treated eyes. This is a positive outcome, especially when we consider that visual acuity is influenced not only by the integrity of the corneal epithelium, but also by other ocular manifestations such as corneal stromal opacification, cataract, glaucoma, and retinal disorders, which cannot be treated by cultivated limbal epithelial cell sheet transplantation. Our study also demonstrated that the degree of corneal neovascularization had improved in 60% and 40% of eyes at postoperative years one and two, respectively, and that the corneal opacity had reduced in 80% of eyes at both time points. Moreover, we encountered no clinically significant adverse events.

The corneal epithelial reconstruction success rate of 60% to 70% found in this trial exceeds the clinically significant success rate of 15% achieved by allogenic limbal transplantation. It also aligns with the findings of Rama et al., who used autologous limbal stem

cells cultivated on fibrin to treat LSCD patients. They found that the success rate after one graft was 68.2%, which after re-grafting increased to 76.6%.[9] These authors also reported that all failures occurred within the first year of grafting, which is similar to our study's findings. The clinical outcomes obtained in the current clinical trial are similar to those reported in published meta-analyses/systematic reviews (Table 23).[10-12]. A significant feature of the current study is the central role of third-party endpoint assessors, who were blinded to any patient information for the photographic/slit-lamp images of the eyes to eliminate any bias.

Previous reports have indicated that autologous cultivated limbal epithelial cell sheets that have a relatively high proportion of stem cells, detected as p63-bright holoclone-forming cells, tend to survive better in the long-term.[9,17] We assessed the percentage of p63-positive cells in our GMP-fabricated cell sheets (Supplemental Table 5). However, we could not correlate this with the likelihood of a successful corneal epithelial reconstruction (data not shown). We also experienced two cases with early postoperative uncontrolled inflammation, and subsequent graft failure and think it likely that the number of stem cells in the cultivated cell sheet and/or the presence of severe early postoperative inflammation can affect graft survival. However, we note that a limitation of the current study is the small number of patients included, which would likely mask any relationship between clinical outcomes and the number of p63-positive cells in the grafted epithelial sheet or the presence of postoperative inflammation. To establish whether either of these factors impact upon clinical outcomes, we are currently conducting post-marketing surveillance of all cultivated limbal epithelial cell sheet transplant surgeries over seven years.

313 In conclusion, autologous cultivated corneal limbal epithelial cell sheet transplantation is
314 a safe and effective treatment for LSCD. A GMP-compliant Cellular and Tissue-based product,
315 named *Nepic*, has been newly approved for ocular regenerative medicine.

316

317 **DATA AVAILABILITY STATEMENT**

318 All data acquired in this clinical trial are available in tables or supplemental files.

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FIGURE TITLES AND LEGENDS

Fig. 1. Transplantation of *ex vivo* expanded autologous corneal limbal epithelial cell sheets

A small biopsy of healthy limbal tissue is obtained from a patient's unaffected eye, after which corneal limbal epithelial stem cells are isolated and cultivated on a temperature-responsive cell-culture dish. Once formed, the *ex vivo* expanded autologous corneal epithelial cell sheet is harvested from the culture dish by lowering the temperature to room temperature, which allows the cell sheet to be picked up and transplanted onto the surface of the diseased eye.

Fig. 2. Eyes before and after transplantation of *ex vivo* expanded autologous corneal limbal epithelial cell sheets

The corneal surface of C-1 and C-2 was successfully reconstructed using *ex vivo* expanded autologous corneal limbal epithelial cell sheets, with corneal transparency improved and maintained. However, conjunctivalization with neovascularization was observed in patient B-3 subsequent to severe early postoperative inflammation.

Table 4. LSCD staging before and after cultivated limbal epithelial cell sheet transplantation

Patient	Age	Sex	Causative disease	Pre-operative	Postoperative						
					2 weeks	4 weeks	12 weeks	24 weeks	52 weeks	78 weeks	104 weeks
A-1	20	F	Chemical burn	IIB	IA	IA	IIA	IA	IA	IA	IA
A-3	79	M	Idiopathic	III	IIB	IA	IB	IIB	IIB	IIB	IIB
B-1	23	F	Idiopathic	IIB	IA	IA	IA	IA	IA	IA	IA
B-2	52	F	VKC	IIB	IA	IA	IA	IA	IA	IA	IA
B-3	83	M	OCP	III	IA	IB	IB	IIB	IIB	IIB	IIB
B-4	38	M	Chemical burn	III	IA	IA	IB	IB	IIB	IIB	IIB
C-1	37	M	Chemical burn	IIB	IA	IA	IA	IB	IIA	IB	IB
C-2	67	M	Chemical burn	III	IA	IA	IA	IA	IA	IA	IA
E-1	42	M	Chemical burn	III	IA	IA	IA	IA	IA	IA	IA
E-2	70	M	Chemical burn	III	IA	IA	IA	IA	IA	IA	IA

LSCD: limbal stem cell deficiency, OCP: ocular cicatricial pemphigoid, VKC: vernal keratoconjunctivitis

Table 6. Postoperative changes in subjective symptoms, visual acuity, corneal opacification, corneal neovascularization, and symblepharon

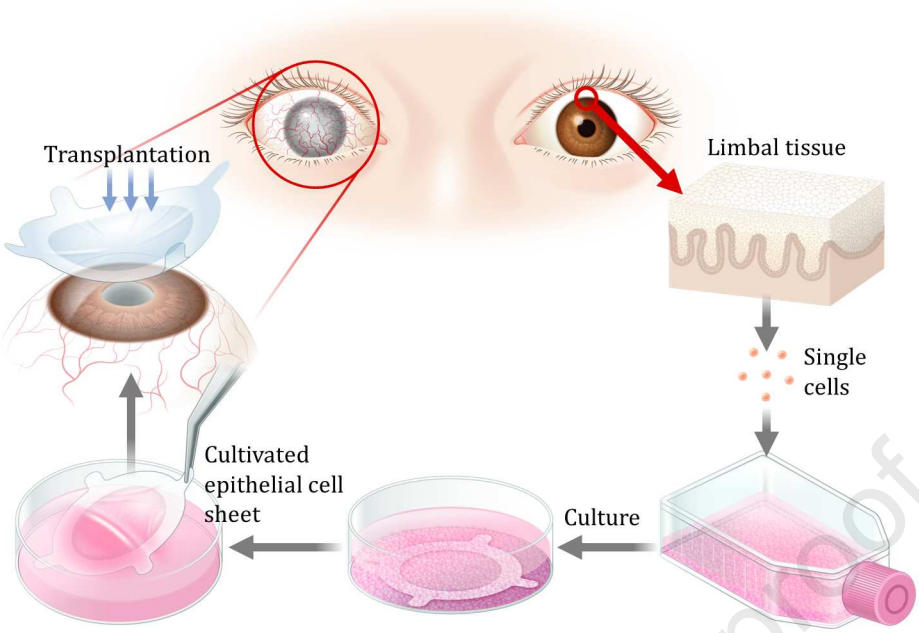
	Ocular pain		Foreign body sensation		Lacrimation		Photophobia		Dryness		Discomfort	
	52 weeks	104 weeks	52 weeks	104 weeks	52 weeks	104 weeks	52 weeks	104 weeks	52 weeks	104 weeks	52 weeks	104 weeks
Improved	0 (0%)	0 (0%)	2 (20%)	3 (30%)	3 (30%)	2 (20%)	3 (30%)	3 (30%)	3 (30%)	2 (20%)	6 (60%)	4 (40%)
Unchanged	7 (70%)	7 (70%)	7 (70%)	5 (50%)	5 (50%)	7 (70%)	6 (60%)	4 (40%)	6 (60%)	5 (50%)	3 (30%)	4 (40%)
Deteriorated	3 (30%)	3 (30%)	1 (10%)	2 (20%)	2 (20%)	1 (10%)	1 (10%)	3 (30%)	1 (10%)	3 (30%)	1 (10%)	2 (20%)

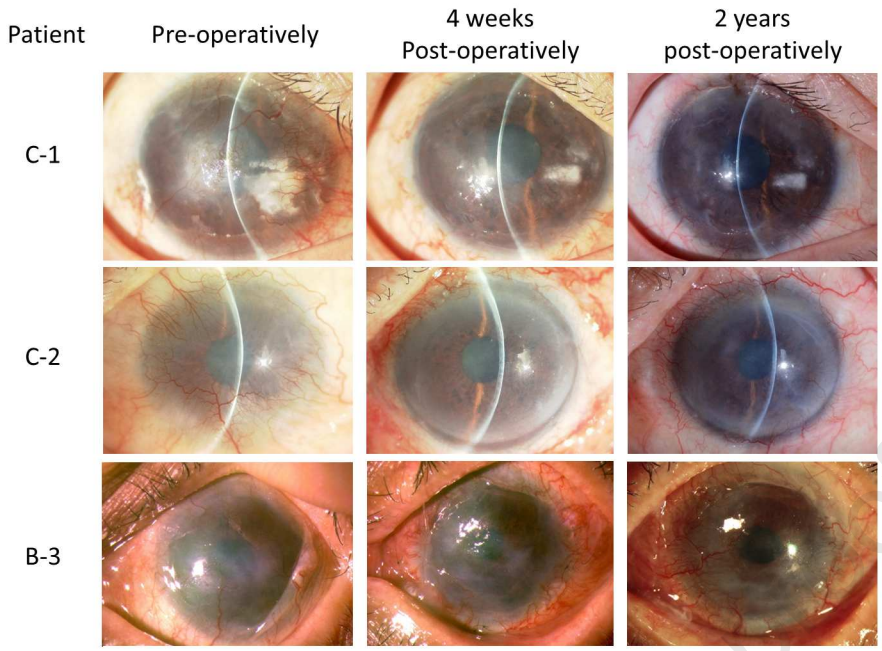
	Decimal visual acuity		ETDRS visual acuity		Corneal opacification		Corneal neovascularization		Symblepharon	
	52 weeks	104 weeks	52 weeks	104 weeks	52 weeks	104 weeks	52 weeks	104 weeks	52 weeks	104 weeks
Improved	6 (60%)	5 (50%)	5 (50%)	6 (60%)	8 (80%)	8 (80%)	6 (60%)	4 (40%)	1 (10%)	1 (10%)
Unchanged	2 (20%)	3 (30%)	3 (30%)	3 (30%)	2 (20%)	2 (20%)	4 (40%)	6 (60%)	8 (80%)	8 (80%)
Deteriorated	2 (20%)	2 (20%)	2 (20%)	1 (10%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (10%)	1 (10%)

ETDRS: Early Treatment Diabetic Retinopathy Study

Table 23. Comparison of clinical outcomes between the current clinical trial and previous systematic reviews and meta-analyses

	Current clinical trial (N=10)	Le Q et al. JAMA Ophthalmology 2020 (Le et al, 2020)	Zhao Y et al. Cornea 2015 (Zhao and Ma, 2015)	Oie Y et al. JJO 2021 (Oie et al, 2021)
Autologous/Allogenic	Autologous	Autologous	Autologous and allogenic	Autologous
Corneal epithelium reconstruction	60% (1 year) 70% (2 years)	84.7%	67%	74.1%
Visual recovery	60% (1 year) 50% (2 years)	56.4%	62%	54.5%
Ocular hypertension	10%	0.3%	-	4.6% (including glaucoma)
Immunological rejection	0%	0%	-	0%
Infectious keratitis	0%	2.1%	-	6.3%





We confirmed the efficacy and safety of autologous cultivated limbal epithelial cell sheet in clinical trials for limbal stem cell deficiency. The cell sheet named *Nepic*, is newly approved as a Cellular and Tissue-based product.