

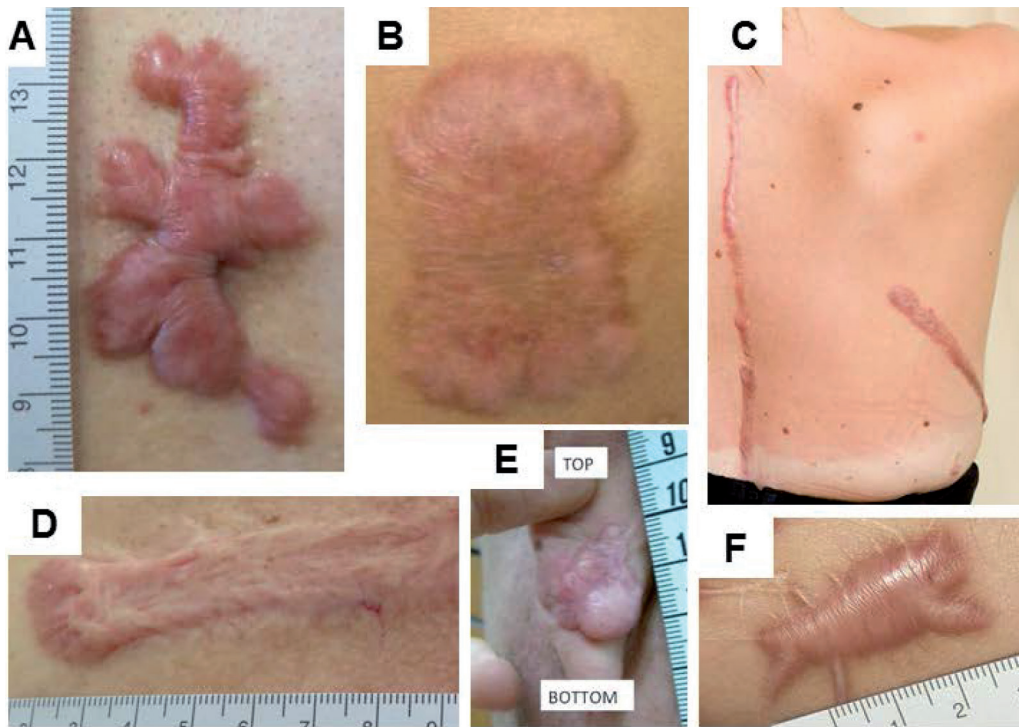


Fig. S1. Clinical variability of representative keloids in the study. A. Sternum. B. Shoulder. C. Trunk. D. Sternum. E. Ear lobe. F. Medial forearm.

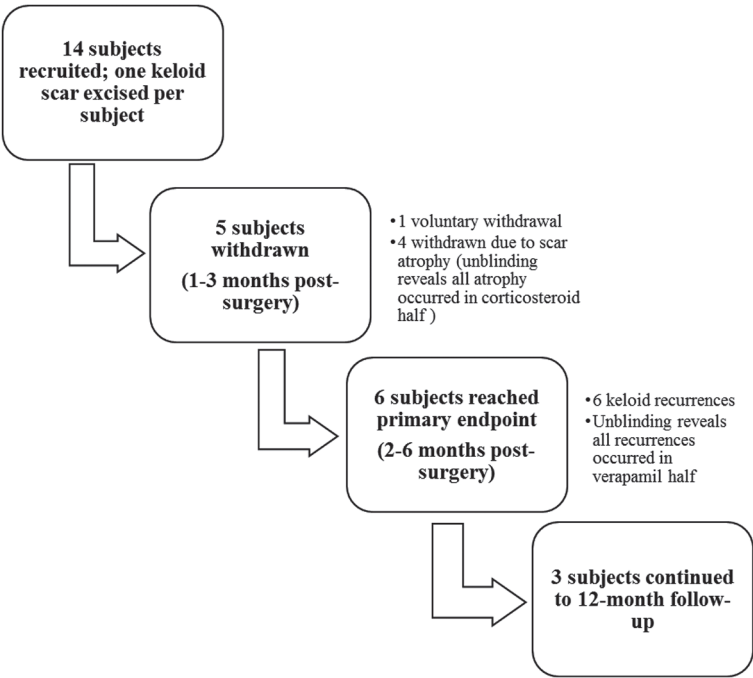


Fig. S2. Diagram summarising subject recruitment and outcomes.

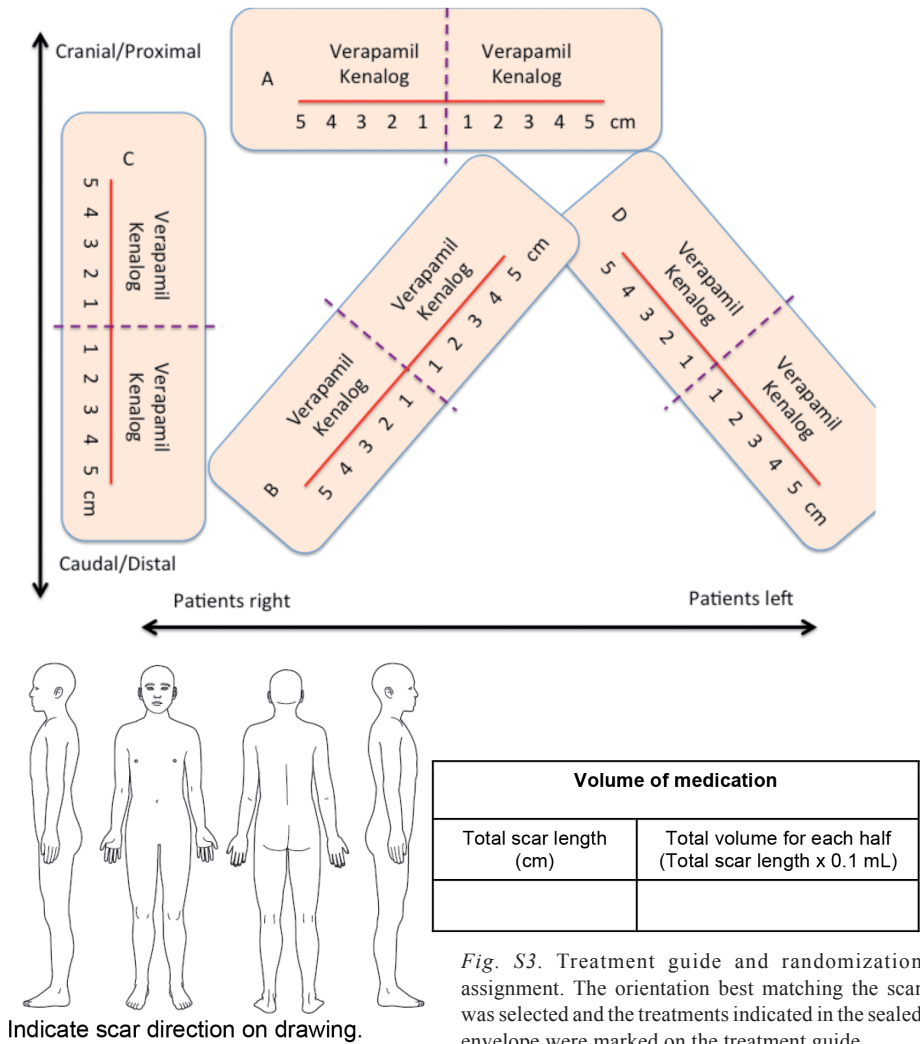


Fig. S3. Treatment guide and randomization assignment. The orientation best matching the scar was selected and the treatments indicated in the sealed envelope were marked on the treatment guide.

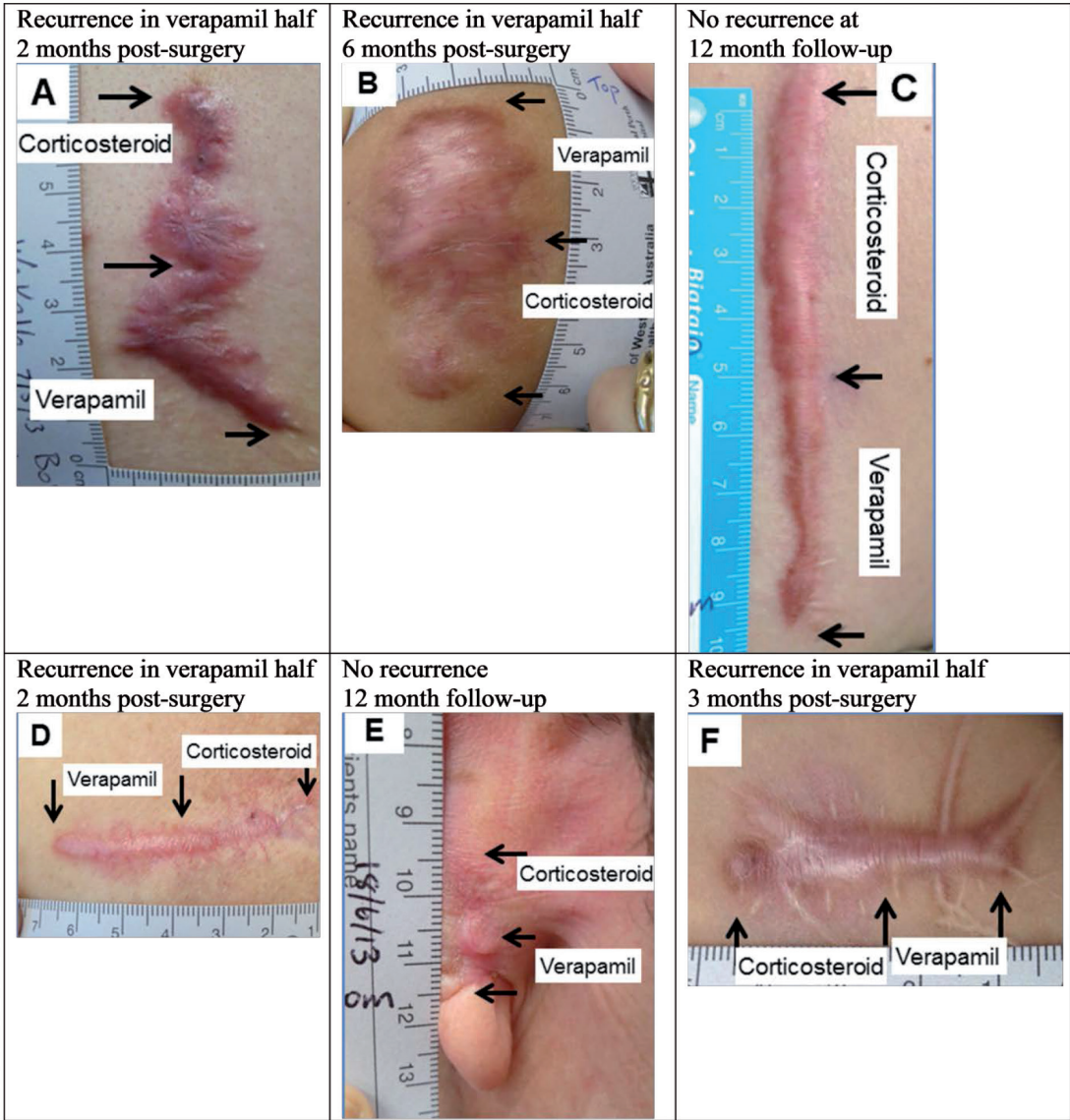


Fig. S4. Outcomes of representative keloids at end point (keloid recurrence) or 12-month follow-up.

Appendix S1

METHODS

Subjects

Adult patients with a keloid scar diagnosed clinically by a plastic surgeon and scheduled for surgical excision at Royal Perth Hospital, Western Australia, were recruited. Participation was voluntary and all patients received the same standard of clinical care whether they elected to participate or not. Inclusion criteria: at least 18 years of age; able to provide voluntary written informed consent; had a (single) keloid scar scheduled for surgical excision which would result in a linear scar 3–10 cm in length. Patients with multiple keloid scars were eligible, but only one scar was to be scheduled for surgical excision during the course of the study. Exclusion criteria: pregnancy/lactation; heart or pulmonary condition; systemic treatment with beta-blockers, ACE-inhibitors, calcium antagonists or steroids; intralesional steroid therapy in last 2 months.

The study followed the guidelines of the Declaration of Helsinki and was approved by the Human Research Ethics Committees of Royal Perth Hospital (EC 2012/067) and the University of Western Australia. All eligible subjects were provided with a patient information sheet and a verbal explanation of the study and written consent was obtained. An independent committee was established to review safety. As the outcome of the study was cosmetically sensitive, a conservative stopping guideline was selected ($p < 0.05$, log-rank test). The study was registered at ClinicalTrials.gov (NCT01720056).

Study design

The study was a randomized controlled trial with a paired split-scar design. Thirty patients were scheduled for recruitment (one scar/patient). Each half of the suture line of the excised keloid was randomly allocated (ratio 1:1) to receive triamcinolone or verapamil using a sealed envelope system (see *Randomization* below and Fig. S1') by authors PLD and HJW. Each half of the scar was assessed 1, 2, 3, 6 and 12 months post-surgery by a scar assessor (UTG) and the subject. Subjects and scar assessor were blinded to the treatment.

Subjects were withdrawn from study if they experienced any side-effects requiring un-blinding of treatment and were completed when the primary endpoint was reached (keloid recurrence).

Study treatments

The active treatments used in the study were Isoptin Injection (verapamil hydrochloride) 5 mg/2 mL (Abbott, Australia) and Kenacort-A 10 Injection (triamcinolone acetonide) 10 mg/1 mL (Aspen, Australia).

Randomization

Sealed envelopes for all planned patients (30) were made prior to study start by PLD. Sequence was generated by random selection of the sealed envelopes and these were marked with consecutive numbers '1' to '30'. Sealed envelopes were opened and scar half treatment allocation revealed to the surgeon immediately after keloid was excised. Thus surgery was performed blinded to active treatment.

Study protocol

Keloid excision was performed while the patient was under general anaesthesia by one surgeon (SMR) using a standardized

technique. All patients had an intralesional excision and two layer closure with monofilament synthetic absorbable surgical suture prepared from a copolymer of glycolide and epsilon-caprolactone (Monocryl™, Ethicon, Johnson & Johnson, New Jersey, USA). No perioperative antibiotics were given. The wound length (3–10 cm) was measured after closure. The test treatments were administered by intradermal injection along each half of the suture/scar line: triamcinolone (2 mg/cm), maximum total dose 10 mg (reduced to 1 mg/cm, maximum total dose 5 mg, after safety review); verapamil (0.5 mg/cm), maximum total dose 2.5 mg. The treatments were injected intradermally immediately post-operatively and at 1, 2 and 3 months post-surgery. The first treatment was performed while the patient was still under general anaesthesia and the follow-up treatments were performed by a plastic surgeon or surgical trainee at the burns unit outpatient clinic, Royal Perth Hospital, under local anaesthesia (lignocaine 1% with epinephrine). Additional scar therapy in the form of topical silicone or pressure garments were permitted when clinically indicated (independent assessment by occupational therapist) as long as these were applied equally to both halves of the scar.

Assessments

Before surgery a photo of the keloid was taken and clinical characteristics of the scar and subject were recorded including skin Fitzpatrick pigmentation type (29), scar location, initiating injury or wound type, scar duration, and scar length and width.

Post-surgical scar assessment was performed on each scar half using clinical examination and the modified Vancouver Scar Scale (mVSS). The modification used was based on Baryza and Baryza (30) with the additional modification of calibrated silicone strips (available in 1 mm, 2 mm and 5 mm thickness) for the measurement of scar height. These strips have been recently developed by Sian Falder and others at the Liverpool Alder Hey Children's Hospital, UK (personal communication). Local and systemic side-effects were evaluated clinically e.g. skin atrophy, changes in pigmentation, pain, pruritus and paraesthesia. The middle 1 cm of the scar, at the junction of the two treatments, was excluded from the assessment. The subject also rated each scar half using the patient scale of the Patient and Observer Scar Assessment Scale v2.0 (POSAS) (31).

Pigmentation and vascularity were quantified objectively using the DermaLab Combo® (Cortex Technologies, Denmark), a device to measure skin colour objectively by narrow-band spectrophotometry. The measurements derived were Melanin Index % (MI%) for pigmentation, and Erythema Index % (EI%) for vascularity (32). These measurements are relative to a matched control site (100%).

Outcome measurements

The primary outcome was keloid recurrence. Keloid recurrence was defined as a scar having greater than 2 mm height and lateral growth beyond the wound boundary into uninvolved skin, accompanied by symptoms of pain or pruritis. Secondary outcome measures were local side-effects, mVSS, POSAS (patient scale), vascularity (EI%) and pigmentation (MI%).

Monitoring safety

At each visit the patient was asked to report side-effects. After each treatment the patient was observed for 15 min for any immediate side effects. Study data was examined by an un-blinded independent safety committee on two occasions before study completion: (i) to review all side-effects after 11 included patients had been treated for four months; (ii) to conduct a pre-specified interim analysis after the first 14 patients

were followed up for 12 months, with a stopping guideline of $p < 0.05$ for benefit on the primary end point (keloid recurrence).

Isolation of fibroblasts and keratinocytes from keloid tissue

Primary cultures of fibroblasts ($n = 7$) and keratinocytes ($n = 6$) were established from excised keloid tissue using protocols previously described (33). Fibroblasts were cultured in DMEM/F-12 with GlutaMAX™ supplement (Life Technologies, Victoria, Australia), 1.05 mM calcium and 10% fetal bovine serum (FBS) (Bovogen Biologicals, Victoria, Australia). Keratinocytes were cultured in serum-free EpiGRO™ Human Epidermal Keratinocyte Complete Medium (Millipore, Temecula, CA, USA) with 60 mM calcium. All media contained 1% penicillin/streptomycin (Life Technologies, Victoria, Australia) and cells were grown in 5% carbon dioxide at 37°C. Five thousand cells from the second passage were cultured overnight on glass coverslips in Cellstar® 24-well plates (Greiner Bio-one, Austria) in 1 ml of media before the functional assessment of L-type calcium channels.

Functional assessment of L-type calcium channels – Intracellular calcium assays

Intracellular calcium in fibroblasts and keratinocytes isolated from the excised keloid tissue was monitored using the fluorescent indicator Fura-2 AM (1 μ M, ex 340/380 nm, em 510 nm; Molecular Probes) at 37°C in HEPES-buffered saline (HBS) containing (in mM): 140 NaCl, 5.4 KCl, 2.5 CaCl₂, 0.5 MgCl₂, 5.5 HEPES and 11 glucose (pH 7.4) as previously described (34). Fluorescent images were taken at 1 minute intervals with 50 ms exposure. Fluorescent signal was measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Metamorph 6.3 was used to quantify the signal by manually tracing cells. An equivalent region not containing cells was used as background and was subtracted. Ratiometric 340/380 nm fluorescence was recorded before and after exposure of cells to either the specific L-type calcium

channel agonist, 2 μ M BayK(-) (Bay K8644, Sigma), or the antagonist, 1 μ M verapamil hydrochloride (Isoptin® Injection, Abbott, Australia). Ratiometric 340/380 nm fluorescence recorded over the final 5 min of each 10 minute exposure was averaged and alterations in fluorescent ratio were reported as a ratio of the baseline average. Pre-treatment 340/380 nm fluorescence was assigned a value of 1.0.

Data analysis

Kaplan-Meier survival curve analysis was undertaken to compare keloid recurrence-free survival in the two treatment groups (log-rank [Mantel-Cox] test and hazard ratio [HR]). The number of scars treated with each drug was used as the denominator in each group. Secondary scar outcomes by treatment in subjects with and without keloid recurrences were analysed by the Wilcoxon sign rank test and side-effects were compared qualitatively between the two treatments. The 340/380 nm fluorescence ratios in keloid cells in response to the L-type calcium channel agonist, BayK(-), and antagonist, verapamil, were analyzed within patients using the Mann Whitney test; responses between treatment groups were compared qualitatively. Statistical analysis was performed using GraphPad Prism, Ver. 5.0. The level of statistical significance was $p < 0.05$.

Sample size calculation

The sample size required for this study was based on the primary outcome, keloid recurrence-free survival. The sample size calculation for the log-rank test in Kaplan-Meier survival curve analysis was performed using the online calculator, StatsToDo (www.statstodo.com/SSizSurvival_Pgm.php). Keloid recurrence-free survival at a 1-year follow-up with surgical excision and post-operative triamcinolone is expected to exceed 80% (35). Type-1 error was set at 5%; to achieve 80% power to detect a decrease in keloid recurrence-free survival from 80% to 40% at least 27 scars in each group were needed.

Table SI. Subject demographics and clinical characteristics of the excised keloid scars

Sex, <i>n</i> (%)	
Female	6 (43)
Male	8 (57)
Age, years, mean $\pm$ SD	32.1 $\pm$ 10.5
Maximum	53
Minimum	18
Fitzpatrick skin type, <i>n</i>	
III	9
IV	3
V	2
Scar duration, years, mean $\pm$ SD	12.0 $\pm$ 10.9
Maximum	36
Minimum	1
Aetiology, <i>n</i>	
Surgical wound	3
Acne	5
Immunization	2
Non-burn trauma	3
Burn	1
Scar location, <i>n</i>	
Sternum	6
Shoulder	3
Back	1
Neck	1
Ear	1
Upper arm	1
Forearm	1
Scar area excised, mean $\pm$ SD	12.6 $\pm$ 14.0 cm ²
Maximum	55 cm ²
Minimum	1.2 cm ²