ORIGINAL ARTICLE

Verapamil-containing silicone gel reduces scar hypertrophy

Jangyoun Choi[1] | Yu Na Han[1,2] | Eun Young Rha[3] | Hwi Ju Kang[4] |
Ki Joo Kim[1] | Il Kyu Park[4] | Hyun-Jung Kim[5] | Jong Won Rhie[1,2]

[1]Department of Plastic and Reconstructive Surgery, College of Medicine, The Catholic University of Korea, Seoul, South Korea

[2]Department of Biomedicine & Health Sciences, College of Medicine, The Catholic University of Korea, Seoul, South Korea

[3]Department of Plastic and Reconstructive Surgery, Eunpyeong St. Mary's Hospital, The Catholic University of Korea, Seoul, South Korea

[4]Genewel Co., Ltd., Sagimakgol-ro 62 beon-gil Jungwon-gu, Seongnam-si, Gyeonggi-do, South Korea

[5]T&R Biofab, Executive Director/Research Director, Regenerative Medicine Lab, Republic of Korea

Correspondence
Jong Won Rhie,Professor, MD, PHD, Department of Plastic and Reconstructive Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222 Banpo-Daero, Seocho-gu, Seoul, 06591, South Korea.
Email: rhie@catholic.ac.kr

Abstract

A hypertrophic scar is a common dermal fibroproliferative lesion usually treated with topical silicone. Verapamil, a type of calcium channel blocker, is considered a candidate drug for the treatment of hypertrophic scars. Here, we report that the addition of verapamil to topical silicone gel enhances treatment outcomes of hypertrophic scars. Upon creation of hypertrophic scars with the rabbit ear model, varying concentrations of verapamil-added silicone gel (0.1, 1, and 10 mg/g) were applied daily for 28 days. After the animals were euthanised, microscopic measurement was performed for (a) scar elevation index (SEI), (b) fibroblast count, and (c) capillary count. On gross analysis, features of hypertrophic scars were significantly alleviated in the verapamil-added groups. On histologic examination, verapamil-added groups showed (a) reduced SEI (1.93 (1.79-2.67) for control vs 1.34 (1.21-1.51) for silicone only and 1.13 (1.01-1.65) for verapamil-added silicone), (b) fibroblast count 700.5 (599.5-838.5) for control, 613.25 (461-762.5) for silicone only, and 347.33 (182.5-527) for verapamil-added silicone), and (c) capillary formation (52 (35.5-96.5) for control, 46 (28-64.5) for silicone only, and 39.83(24-70) for verapamil-added silicone) (Kruskal-Wallis test, $P < .05$). On western blot, expression levels of collagen I protein was lower in the 1 mg/g and 10 mg/g verapamil-added silicone compared with control. Therefore, we suggest a therapeutic concentration of verapamil-added silicone gel of at least over 1 mg/g. Further study regarding maximally effective concentration and deeper insight into the mechanism of action should follow.

KEYWORDS
calcium channel blocker, collagen, scar, silicone, verapamil

1 | INTRODUCTION

Hypertrophic scar is a common dermal lesion caused by abnormal fibrosis after surgery or trauma. It is functionally and cosmetically concerning because of its elevated, hypervascular, pruritic, and inelastic nature. These features develop from the hypertrophic wound matrix, which shows immature, disoriented collagen deposition and excessive capillary formation.[1,2]

Over the past several decades, various approaches for improving the cosmesis of hypertrophic scars have been suggested. Widely accepted non-invasive

modalities include occlusive therapy with silicone sheet or gel, scar hydration, compression therapy, intralesional injection, and laser and surgical excision.[3-7] Despite the diversity of treatment modalities, the outcome is inconsistent, and a better treatment agent is still required.[8]

Verapamil, a widely known calcium channel blocker, is considered a candidate drug for the treatment of hypertrophic scars. Because calcium-related cellular activity is ubiquitous, applying verapamil to non-vascular tissue can alter its physiology.[9] As the activity of fibroblasts is also calcium-related, it is reasonable to use verapamil for the treatment of hypertrophic scars.

Intralesional injection of verapamil was reported as an effective therapy for scar treatment.[10] However, current high-level evidence suggests intralesional therapy is best reserved for agents such as triamcinolone or botulinum toxin.[11] In this context, local, topical delivery of verapamil for scar treatment is more favourable because it does not result in a systemic hypotensive effect, and it saves treatment opportunities for intralesional injection in other therapies. Therefore, in this study, we sought to affirm the effectiveness of verapamil using a different mode of delivery. Conventional silicone gel was used not only as a treatment agent itself but also as a sustained verapamil-releasing platform. Multiple concentrations of verapamil were tested on a standardised, hypertrophic scar model created on rabbit ears. The degree of improvement was then quantitatively analysed.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Ten male New Zealand white rabbits aged 5 months or older and weighing 2500 to 3000 g were used in this study. All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee of the Catholic University of Korea, School of Medicine (CUMC-2016-0136-02). The rabbits were kept in separate cages at room temperature (22-24° C) with relative humidity of 40 to 60% on a 12-hour light/dark cycle. Food and water were provided as needed.

2.2 | Scar model establishment

We used a widely accepted hypertrophic scar model suggested by Mustoe et al. Rabbits were anaesthetised with 15 mg/kg of Zoletil (Virbac, Carros, France) and 5 mg/kg of Xylazine (Bayer, Leverkusen, Germany) by intramuscular injection. Routine hair removal and

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Key Messages

- Addition of verapamil microparticles further enhances the scar-reducing effect of silicone gel.

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aseptic preparation were performed. Five punch wounds (diameter = 8 mm) were created on the ventral side of each ear using a skin biopsy punch (10 wounds/rabbit, total of 100 wounds). Wounds were arranged in a pentagonal formation, at least 1.5 cm from each other to prevent interaction between treatments. Careful dissection under magnification was performed to remove skin and perichondrium to expose the bare cartilage. Care was taken to ensure that the perichondrial layer was completely removed. Wounds were covered with transparent polyurethane dressing (OpSite; Smith and Nephew, Andover, Massachusetts) (Figure 1, day 0).

Animals were inspected daily for signs of wound complications. Photographs were produced weekly to evaluate epithelialisation and scar hypertrophy. By day 17, all wounds showed epithelialisation, and dressings were removed at that point. By day 28, all 100 wounds developed into hypertrophic scar lesions that show distinctive raised, red, and hard morphologic features (Figure 1, day 28).

2.3 | Production of verapamil-containing silicone gels

Verapamil-containing silicone gels were produced by mixing varying amounts of verapamil hydrochloride with silicone resin (Dow Corning, Midland, Michigan) to achieve concentrations of 0.1, 1, and 10 mg/g of verapamil-embedded silicone. Distilled water and routine emollients were added.

2.4 | Treatment group allocation

Each wound per ear was assigned to one of five treatment groups: negative control (no treatment), positive control (silicone gel without verapamil), and three treatment groups of verapamil-containing silicone gel at increasing concentrations (0.1, 1, and 10 mg/g of verapamil embedded in silicone gel). The treatment group was sequentially assigned, and the formation was rotated in a clockwise direction ear by ear to evenly distribute the location of the specimen to each treatment group.
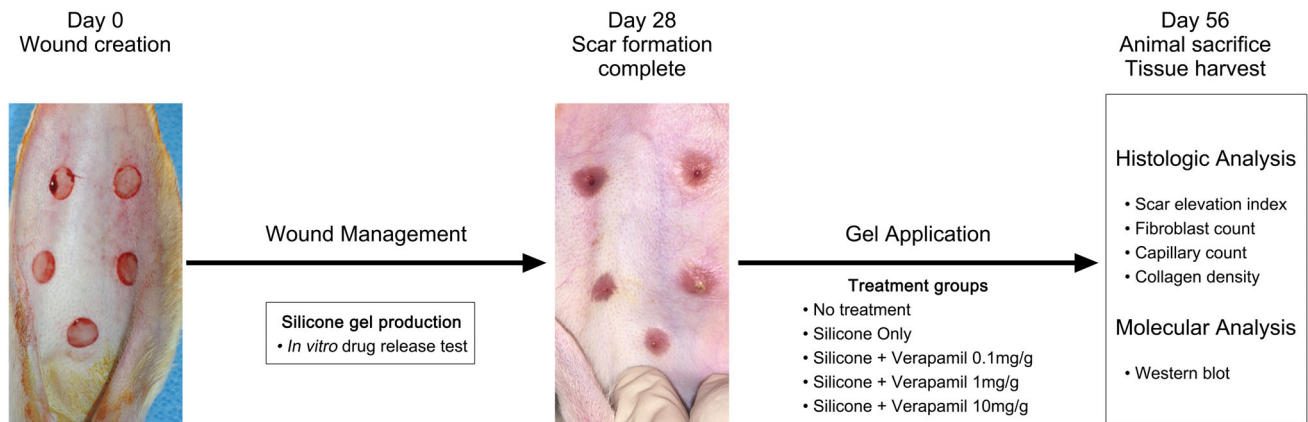
FIGURE 1 Study outline. Eight-millimetre punch wounds were created on each ear of 10 New Zealand rabbits. After 28 days of care, all wounds developed into hypertrophic scars. Scars were evenly distributed into five groups, including a control, and were treated or not with various concentrations of verapamil-loaded silicone gel. After 4 weeks of treatment, the animals were euthanised and underwent analysis

2.5 | Verapamil application

Upon complete formation of hypertrophic scar, drug application was conducted until day 56. The negative control group (no treatment) was merely observed. The other four groups were treated daily with 1 gram of the designated drug. The drug was gently applied with a cotton tip applicator under minimal pressure to avoid the effect of scar massage. A wide plastic neck collar was placed on the rabbits to prevent ear manipulation. During application, rabbits were observed for any signs of drug-related complications such as dermatitis, rash, maceration, or infection.

2.6 | Tissue harvest

After 4 weeks of drug application (day 56), all animals were euthanised, and specimens were harvested with 0.5 cm of normal tissue around the scar. Specimens were then divided into halves at the point of maximal height. One half of each specimen was fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections, and stained using hematoxylin and eosin (H&E) and Masson's Trichrome stain. The other half was processed for western blot analysis (Figure 1, day 56).

2.7 | Gross morphology and microscopic histomorphometry

H&E sections were digitally scanned and quantitatively measured using a Pannoramic MIDI II automatic slide scanner and a Pannoramic Viewer, respectively (both manufactured by 3DHISTECH, Budapest, Hungary). Widely accepted scar-related parameters of (a) scar elevation index (SEI), (b) fibroblast count, and (c) capillary count were quantitatively measured. SEI is derived from the cross-sectional area of a scar as the ratio of total area (hypertrophied tissue and normal tissue underneath) to normal tissue area[12] (File S1). An SEI value approximating one indicates minimal hypertrophy of the lesion, resembling the height of normal tissue. Fibroblast count was measured under x400 magnification in a random field of 1mm². Capillary count was measured under x40 magnification.

2.8 | Relative collagen density

To compare the amount of collagen deposition between treatment groups, relative collagen density was calculated with Masson's Trichrome-stained slides. First, the collagen component was segmented by color channel filtering and area segmentation with ImageJ.[13] The density of the collagen component was measured as the percentage area of the blue-stained region in a chosen field. Relative collagen density was calculated by normalising the collagen density of the scar region to that of the adjacent normal region.

2.9 | Western blot

Total protein was separated by 5% and 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (BIO-RAD, Seoul, Korea), and blocked in 5% skim milk. Non-specific proteins were blocked by incubating the membrane in blocking buffer (5% skim milk in tris-buffered saline and Tween-20) and incubated with the primary antibodies COL1 (1:1000; Novus Biologicals, Littleton, Colorado) and anti-MMP-1 (MAB3307 1:400; MilliporeSigma, Burlington, Massachusetts) overnight at 4°C. The membranes were incubated with the secondary antibody for 2 hours. Protein expression was visualised using the

luminal-enhanced chemiluminescence detection kit (BIO-RAD). Densitometrical signals of the blot were captured and quantified (LAS 4000; Fujifilm, Tokyo, Japan).

2.10 | Drug release test

The verapamil-loaded silicone gels were tested for drug release. They were applied onto a semipermeable membrane through which verapamil could cross over and dissolve into the phosphate-buffered saline (PBS) tank underneath. While the drug was spontaneously released across the membrane, 1 cc of PBS was sampled and measured at increasing intervals (1, 2, 4, 6, 9, 12, and 24 hours) and was replaced with a fresh 1 cc after each sampling. Analysis was conducted using high-performance liquid chromatography.

2.11 | Comparison with conventional products

Three rabbits (10 wounds per rabbit, total of 30 wounds) were separately prepared for comparison with products currently in the market. The scar model described above was created in the same fashion. A dose of 1 mg/g of verapamil plus silicone gel and conventional products (silicone gel only, heparinised onion extract) were

applied, along with a control group. After obtaining tissue samples, the SEI was evaluated.

2.12 | Statistical analysis

All quantitative measurements were performed by two blinded examiners, and the values were averaged. The results were subjected to the Kruskal-Wallis test. For post hoc comparison, the pairwise Dunn test was conducted with Holm-Bonferroni adjustment to control familywise error rates. All data were analysed with R statistical software, version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria). A value of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Gross morphology

After 28 days of development, all wounds transformed into typical hypertrophic scars that exhibited red, hard, and raised skin inside the original wound boundary (Figure 2). During drug application from days 28 to 56, treatment groups with added verapamil showed meaningful loss of redness and volume. At treatment endpoint (day 56), the control group
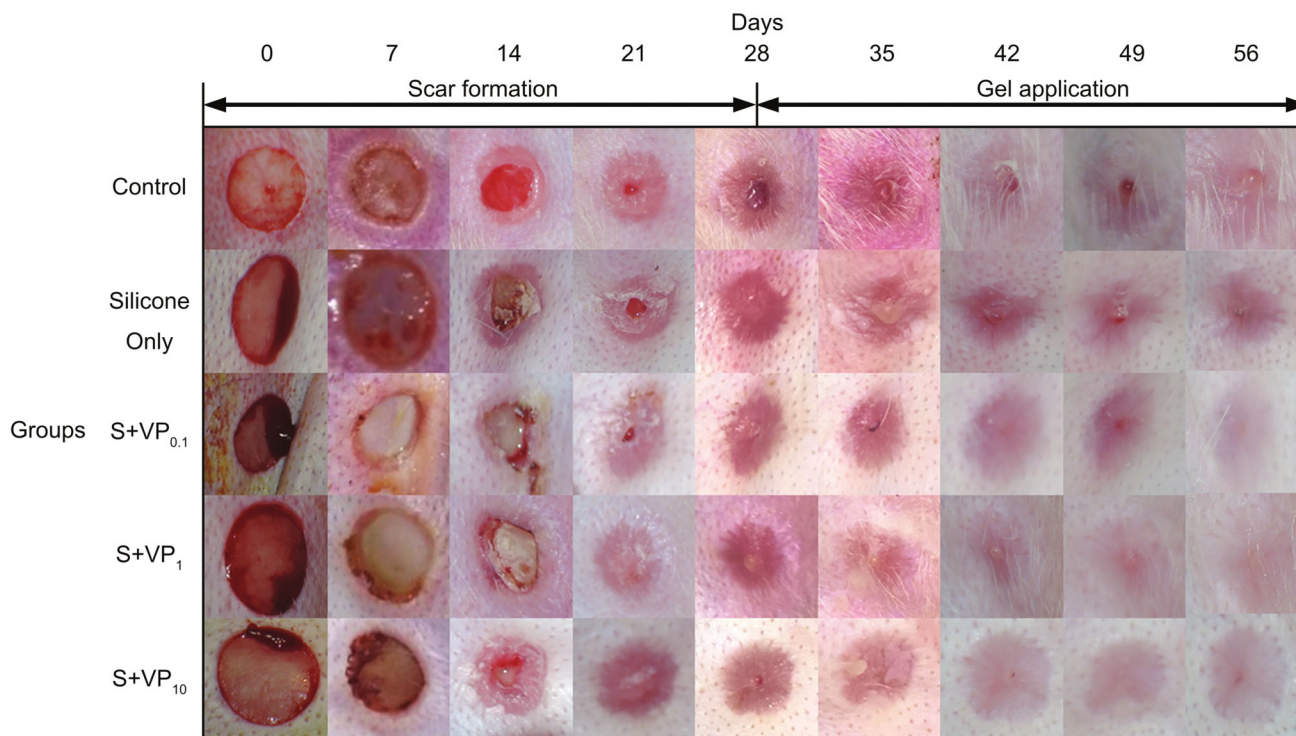

FIGURE 2 Weekly photographs of scars from initial surgery to treatment endpoint. S + VP0.1, silicone plus 0.1 mg/g verapamil. S + VP1, silicone plus 1 mg/g verapamil. S + VP10, silicone plus 10 mg/g verapamil
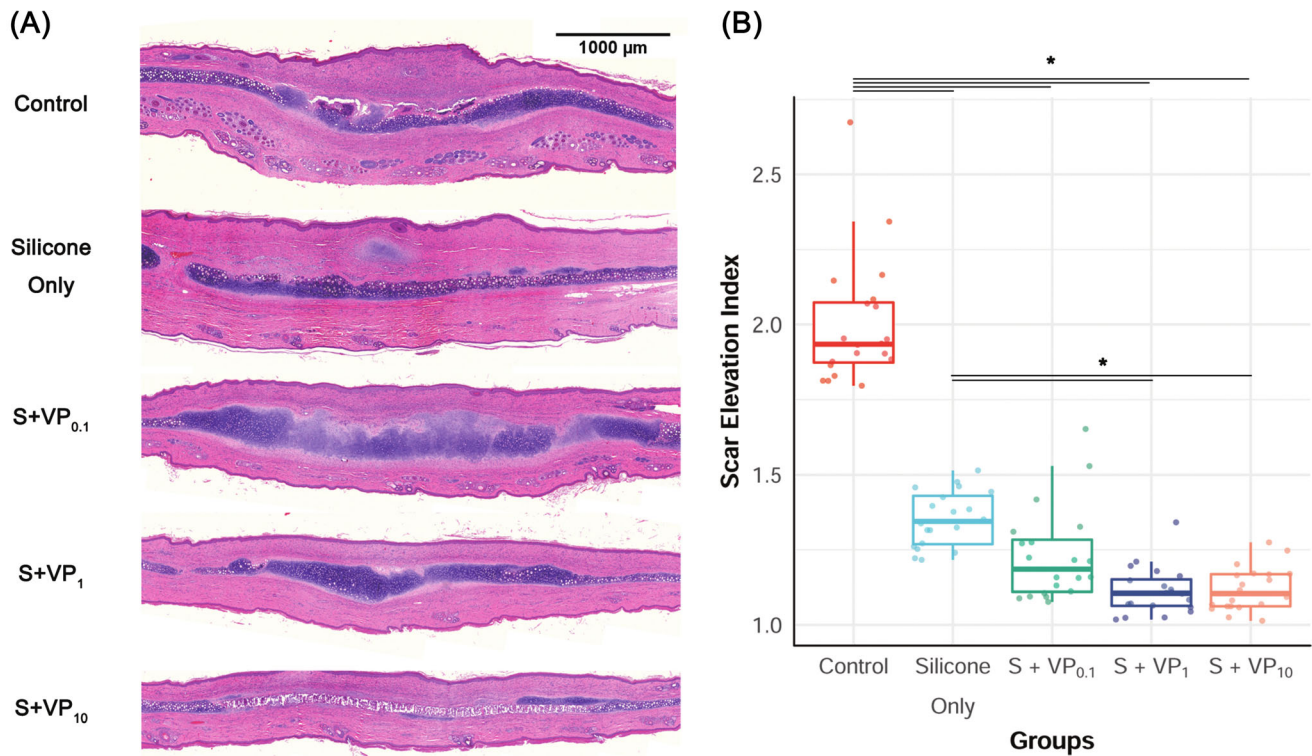
FIGURE 3 Change of scar height among control, silicone only, and verapamil-added silicone groups. (A). Histology under low-power field light microscopy (x20). (B). Quantitative measurement of scar elevation index with digital histomorphometry. Asterisks denote statistical significance ($P < .05$) by the pairwise Dunn test after the Kruskal-Wallis test. S + VP0.1, silicone plus 0.1 mg/g verapamil. S + VP1, silicone plus 1 mg/g verapamil. S + VP10, silicone plus 10 mg/g verapamil

demonstrated scar-related features, for example, raised skin contour and redness, despite the natural scar remodelling process. The silicone-only group showed modest reduction in elevation and redness. The verapamil-added groups exhibited significantly flatter scar volume and less erythema than both the control and silicone-only groups.

3.2 | Microscopic histomorphometry with SEI

On low-power field microscopy, a gradual decline in gross scar height from the control group to verapamil-added groups was observed (Figure 3A and Table 1). Quantitative measurement of SEI followed by a Kruskal-Wallis test confirmed at least one statistical difference between any two treatment groups (Figure 3B, $P < .001$). On post hoc pairwise comparison, a significant difference in SEI was observed between the control group and all other treatment groups. Pairwise comparison between silicone-only treatment and 0.1 mg verapamil with silicone treatment failed to show significance

($P = .053$). However, addition of 1 and 10 mg/g verapamil to silicone gel showed significantly reduced SEI compared with silicone-only treatment ($P < .01$ on both analyses).

3.3 | Microscopic histomorphometry with fibroblast count

A significant difference in fibroblast count was found among treatment groups (Figure 4A and Table 1, $P < .01$). Post hoc pairwise comparison did not show a significant difference in fibroblast count between the control and silicone-only groups ($P = .11$). However, comparisons between the control and the three verapamil-containing silicone groups showed statistical significance ($P < .01$). Comparison between the silicone-only group and the verapamil-containing silicone groups showed statistical significance compared with the silicone plus 0.1, 1, and 10 mg/g of verapamil groups ($P < .01$). On high-power magnification, decrease of cellularity in verapamil-added groups was noted (Figure 4B).

TABLE 1 Data summarisation

Groups	Scar elevation index	Fibroblast count	Capillary count	Collagen index
Control	1.93 (1.79–2.67)	700.5 (599.5–838.5)	53 (35.5–96.5)	0.92 (0.60–1.05)
Silicone only	1.34 (1.21–1.51)	613.25 (461–762.5)	46 (28–64.5)	0.85 (0.65–1.32)
Silicone + verapamil 0.1 mg/g	1.18 (1.07–1.65)	415.75 (310–527)	44.25 (26.5–70)	0.72 (0.57–1.00)
Silicone + verapamil 1 mg/g	1.11 (1.01–1.34)	364.25 (286.5–415)	39 (26–57.5)	0.72 (0.53–0.96)
Silicone + verapamil 10 mg/g	1.10 (1.01–1.27)	262 (182.5–428.5)	36.25 (24–53.5)	0.74 (0.55–0.98)

Note: Values are presented as median (minimum-maximum).
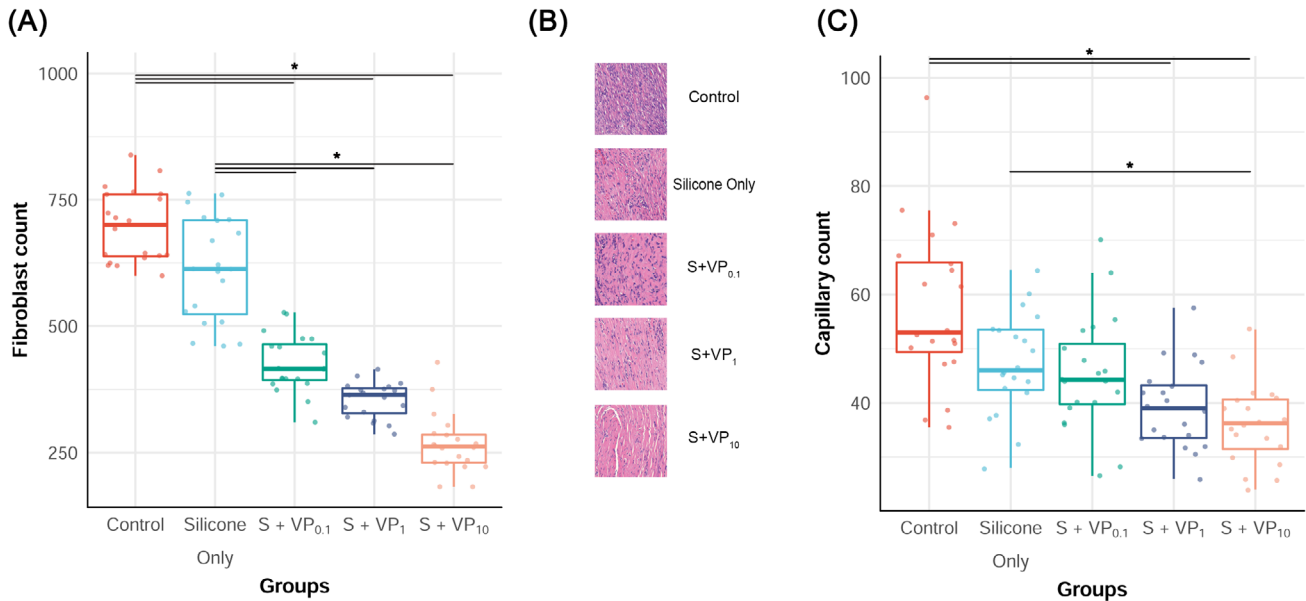

FIGURE 4 Histologic analysis of scar-related features in verapamil-added silicone treatment groups. (A) Fibroblast count within a random field of 1 mm^2. (B) Tissue cellularity and vascularity from control group to silicone plus verapamil groups in a high-power field (x400). (C) Number of capillaries within a random field of 1 mm^2. Asterisks denote statistical significance ($P < .05$) by the pairwise Dunn test after the Kruskal-Wallis test. S + VP0.1, silicone plus 0.1 mg/g verapamil. S + VP1, silicone plus 1 mg/g verapamil. S + VP10, silicone plus 10 mg/g verapamil

3.4 | Microscopic histomorphometry with capillary count

Significant differences were found among treatment groups (Figure 4C and Table 1, $P < .01$). On post hoc pairwise comparison, statistical significance was found between the control and two verapamil-containing (1 and 10 mg/g) groups ($P < .01$). In contrast, comparisons between the control and silicone-only group and between the control and 0.1 mg/g verapamil-containing silicone group failed to show significance ($P = .12$). Comparison between the silicone-only and verapamil groups showed statistical significance between the silicone-only group and the 10 mg/g verapamil-containing silicone group ($P = .018$). On high-power magnification, a decrease in the number of capillaries was evident (Figure 4B).

3.5 | Relative collagen density

The Kruskal-Wallis test confirmed significant differences among treatment groups (Figure 5A and Table 1, $P < .01$). Compared with the control group, the silicone-only group did not show statistical significance ($P = .27$). However, all verapamil-containing groups showed statistical significance ($P < .01$). Compared with the silicone-only group, only the 1 mg/g verapamil-containing group showed statistical difference ($P = .016$).

3.6 | Western blot

Western blot was conducted for Collagen I (COL1) and Matrix metalloproteinase (MMP-1) proteins. Protein expression levels normalised to internal controls
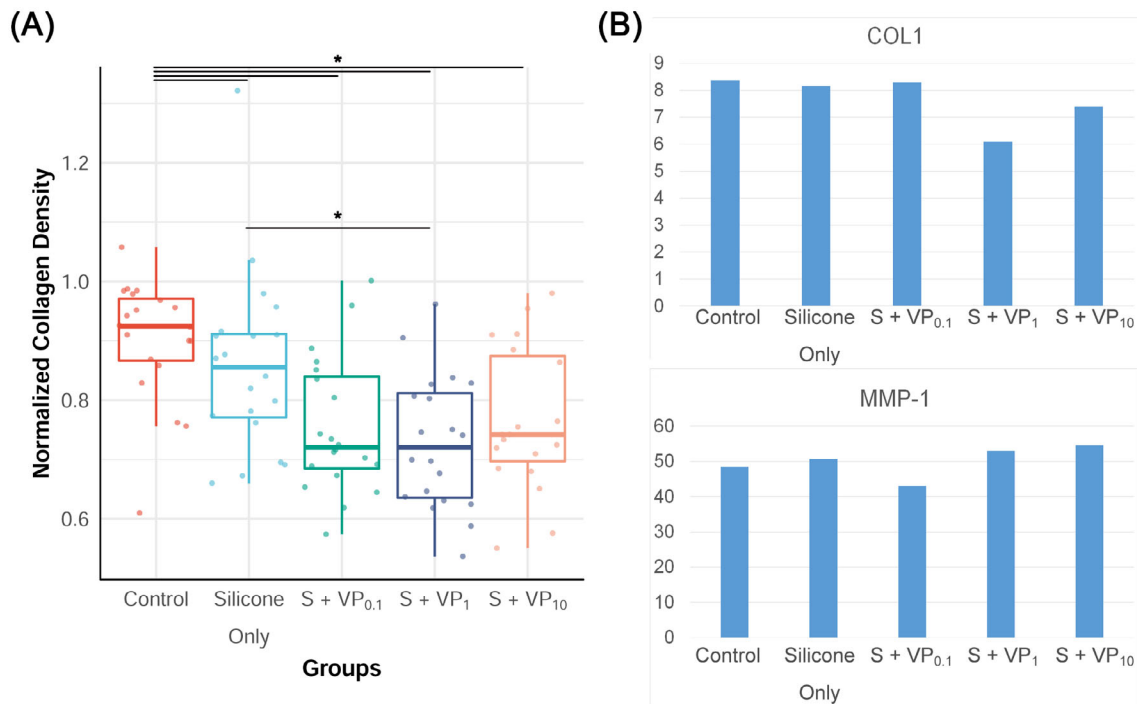
FIGURE 5 Collagen density and Western blot. (A) Density of collagen in scar area normalised to adjacent normal tissue. (B) Western blot of COL1 and MMP-1 for each group. Asterisks denote statistical significance ($P < .05$) by pairwise Dunn test after Kruskal-Wallis test. S + VP0.1, silicone plus 0.1 mg/g verapamil. S + VP1, silicone plus 1 mg/g verapamil. S + VP10, silicone plus 10 mg/g verapamil

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were calculated (Figure 5B). Decreased expression of COL1 was found in both the 1 and 10 mg/g verapamil-combined silicone gels, as was increased expression of MMP-1. However, the results did not show statistical significance ($P = .064$ and 0.14 for COL1 and MMP-1, respectively).

3.7 | Drug release test

Upon drug release testing, a total of 0.089 μg/g of verapamil was detected from the 24-hour release of 0.1 mg/g verapamil with silicone gel. In the same test, 0.15 and 0.73 μg/g of verapamil were detected from 1 to 10 mg/g of verapamil with silicone gel, respectively. A time-serial plot of the samples obtained at 1, 2, 4, 6, 9, 12, and 24 hours showed the steady release of verapamil. No drastic release or loss of verapamil was observed at the early stage (File S2).

3.8 | Comparison with conventional products

Decrease in SEI was found in verapamil-added silicone groups. The Kruskal-Wallis test confirmed significant differences between verapamil plus silicone, control, and conventional products ($P = .001$). On pairwise comparison, about 21% decrease in SEI was found compared with conventional products ($P = .017$, File S3).

4 | DISCUSSION

This study demonstrated the therapeutic effect of a calcium channel blocker combined with silicone gel on hypertrophic scars. Gross morphologic features suggested that the combination of verapamil and silicone improves the overall quality of hypertrophic scars by reducing scar height and redness. This was verified with quantifiable histomorphometric parameters.

The action of verapamil on scar tissue at the cellular level has been shown in previous studies. Upon treatment of verapamil and subsequent decrease of intracellular calcium, the shape of the fibroblast is altered into a spherical shape because of the shortening of the cytoskeleton. This in turn regulates the extracellular secretion of collagenase. In addition, calcium-depleted fibroblasts show a decrease in proliferation and an increase in apoptosis.[14]

Based on this cellular mechanism, the literature shows a few attempts to evaluate the therapeutic effect of verapamil on hypertrophic scars. Intralesional injection of verapamil in a small patient group showed positive results.[15,16] Some studies have investigated the

therapeutic effects of silicone and verapamil but used different methods for different disease entities. Verapamil cream has been studied in the treatment of other fibrotic disorders such as Dupuytren's contracture and Peyroni's disease but not for hypertrophic scars.[17] Previous studies on the treatment of hypertrophic scars focussed on the use of verapamil in a direct intradermal injection.[10,16] Although one study used topical verapamil as a mixture of the drug with non-ionic gel, it was not substituted for a gel with an active ingredient such as silicone.[18] Contrary to the proven potential of verapamil, the groundwork for its use in hypertrophic scars, especially in determining the minimum effective concentration and the best route of administration, is rudimentary. Therefore, we looked into the little-known possibility of scar improvement by combining the two active elements.

Because verapamil is hydrophilic, it is difficult to achieve dissolution in a hydrophobic medium such as silicone. We observed phase separation of verapamil from the silicone media when the concentration of verapamil exceeded 10 mg/g. Hence, 10 mg/g was determined as the maximum concentration. Lower concentrations were considered to be sequential 10-fold decreases from the maximum (1 and 0.1 mg/g).

The achievement of transdermal delivery is the key premise of this work. In this context, selecting silicone as the release medium for transdermal delivery poses merit. The occlusive effect of silicone provides an overhydrated epidermis than a normal epidermis. Hydration changes the cellular morphology of the stratum corneum into a rounder shape, which permits increased transfer of drug molecules into deeper layers.[19] All major routes of transdermal drug delivery, namely, transcellular, intercellular, and transappendegeal, are enhanced by this change in the epidermal environment.[20-22] The transdermal drug release model showed steady transfer of verapamil across the semipermeable membrane over 24 hours (File S2). As the stratum corneum layer is considered a major hurdle for transdermal drug delivery, promoting transfer across this cellular barrier using silicone media is advantageous for transdermal drug delivery.

From a clinical perspective, we believe that the best way to administer verapamil to a scar patient is through a transdermal route by means of silicone loading. Oral verapamil is not a good choice because of its effect of lowering blood pressure. Intralesional injection of verapamil is also suboptimal because of the required frequency for injections. Topical silicone gel combined with verapamil does not lead to systemic hypotension, is convenient to apply, and shows enhanced results. Moreover, it does not preclude additional treatment modalities because there is no separate procedure necessary for administration.

The most important prerequisite of this experiment is a reliable animal model that mimics human hypertrophic scars. First suggested by Morris and colleagues, the rabbit ear model implemented in this experiment satisfies this premise.[2] This model has been adopted in numerous studies and is the most accessible and predictable animal model to simulate human hypertrophic scars.[12,23] Likewise, we were able to successfully reproduce a scar model like those created in the literature.

In addition, our procedure for drug production and application to the scar was validated by reproducing the long-proven effect of silicone on hypertrophic scars. As reported in numerous studies, the silicone-only treatment group in our experiment exhibited significantly reduced median SEI compared with that of the control group. Finally, another precondition of this study is stable emission of verapamil from silicone gel. Time-serial results from our drug release test showed that verapamil is constantly released from silicone.

Scar volume is directly related to collagen abundance. In this regard, the rationale of incorporating calcium channel blockers for treatment of hypertrophic scars is based on numerous in vitro studies that showed the relationship between verapamil and collagen production. They demonstrated diverse modifications of fibroblast activity towards reduced scarring upon verapamil treatment, which was well corroborated with the current experiment. Multiple studies have claimed that, when cultured fibroblasts are treated with verapamil, they show decreased production of collagen, increased production of matrix metalloproteinase, and increased rate of apoptosis.[14,24] Accordingly, we observed decreased median SEI, fibroblast count, and collagen density in all verapamil-added treatment groups. Furthermore, the reduction of scar volume was sufficient enough to be indicated in gross examination. Although not statistically significant, decrease of COL1 and increase of MMP-1 in some verapamil-added groups were observed in western blot results. These results were comparable with silicone-only groups, which strongly suggests an additional effect of verapamil.

Capillary overexpression is responsible for red discoloration of hypertrophic scars. On gross examination, a decrease of erythema was observed in verapamil-added groups compared with the control or silicone-only group. However, only the 10 and 1 mg/g verapamil-added silicone groups showed significant decrease in capillary count. According to the literature, the effect of verapamil in vessel formation is thought to be flexible, depending on the wound-healing phase of a scar. An in vitro study observed decreased production of vascular endothelial growth factor in verapamil-treated mature scar fibroblasts.[25] Conversely, verapamil is also believed to induce

angiogenesis and promote wound healing in the acute wound environment.[26] The inconsistency of capillary count might be explained by this temporal role change of verapamil within the same wound environment. However, we think verapamil is a valid agent for preventing excessive capillary formation because scar treatments are usually conducted well past the acute wound-healing phase.

In summary, the concentration of verapamil can be inferred at a level capable of regulating fibroblast activity, reducing collagen content, and preventing excessive capillary formation. A feasible concentration of verapamil would be at least 1 mg/g, which concurrently demonstrated decrease of scar height, inhibition of fibroblast proliferation, and reduction of collagen density. The exact safety profile of verapamil administration to the skin has not been established. According to a systematic review of 14 articles, 2.5 mg/mL of verapamil was the safe consensus dosage for direct injection.[27] However, considering the low bioavailability of the topical route, we assume that the concentration can safely be increased to higher than 1 mg/g. A detailed dose escalation study should follow to elucidate a safe yet effective concentration.

This work has led us to conclude that verapamil-releasing silicone gel is effective and is a superior alternative to the conventional silicone gel. We propose 1 mg/g as the optimal concentration of verapamil per silicone gel, which makes it possible to inhibit collagen production and capillary formation simultaneously. Determination of the appropriate concentration and duration for application and comparison with other previously accepted agents are planned for future investigation. The indication for verapamil-loaded silicone should not only focus on the treatment of hypertrophic scars but also include keloid and other fibrotic disorders that share common pathophysiology.

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CONFLICT OF INTEREST
None.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Jangyoun Choi https://orcid.org/0000-0002-5165-8414
Yu Na Han https://orcid.org/0000-0003-4186-940X
Jong Won Rhie https://orcid.org/0000-0002-9398-8059

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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