Research Article

**CO₂ fractional laser-assisted transdermal delivery of silk nanofiber carriers in a rabbit ear hypertrophic scar model**

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**Abstract**

**Background:** Hypertrophic scars are skin fibrotic diseases, characterized by fibroblast hyperproliferation and excessive accumulation of extracellular matrix. However, topical drug application for hypertrophic scars are unsatisfactory. The purpose of this study was to explore the permeability of silk nanofiber hydrogels (SNFs) loaded with rhodamine 6G (R6G) and rhodamine 110 (R110) mediated by CO₂ fractional laser irradiation into hypertrophic scar tissues.

**Methods:** In this work, R6G and R110 were chosen as hydrophilic and hydrophobic model molecules. They were loaded inside SNFs. In vivo rabbit ear hypertrophic scars were treated with CO₂ fractional laser irradiation and then R6G/R110-laden SNFs were applied to the scars to evaluate their synergetic effect on drug penetration efficiency. Their permeability was quantified by fluorescence intensity and measured by confocal laser scanning microscopy on days 1, 3, 5 and 7. More specifically, the thermal coagulation zone (CZ) and its surrounding area (peri-CZ) caused by the thermal coagulation of the laser were discussed separately.

**Results:** Our data indicated that the SNFs promoted the penetration of R6G but not that of R110 in the peri-CZ on day 1 when combined with laser irradiation. Interestingly, both R6G and R110 were abundant in the CZ and remained stable on days 1, 3 and 5. Moreover, rapid re-epithelialization hindered the long-term permeability of both drugs.

**Conclusion:** Combining CO₂ fractional laser irradiation with SNF drug delivery could improve the efficiency of hydrophilic drug delivery within 24 h before total re-epithelialization.

**Key words:** CO₂ fractional laser, Hypertrophic scar, Silk nanofiber hydrogels, Transdermal delivery, Laser-assisted drug delivery

**Highlights**

- Silk nanofiber hydrogels provide a suitable platform for revealing the permeability of different drugs into skin treated with CO₂ fractional laser irradiation due to their good loading capacity for both hydrophilic and hydrophobic drugs.
- CO₂ fractional laser irradiation was shown to enhance the permeability of hydrophilic and hydrophobic drugs in rabbit ear hypertrophic scars.
- Silk nanofiber hydrogels combined with CO₂ fractional laser irradiation could improve the skin penetration of hydrophilic drugs within 24 h after laser treatment (before total re-epithelialization).
Background

Abnormal wound healing usually results in hypertrophic scars (HSs) or even keloids [1, 2]. HSs cause organ dysfunction and motor incapacity, imposing a substantial financial burden on patients worldwide [3, 4]. HS treatment includes excision, radiation therapy and the intraleSIONal injection of steroids, 5-fluorouracil or botulinum toxin A [5]. However, researchers are still searching for more effective and convenient HS treatment [6, 7]. Surgical incisions are invasive procedures that are likely to result in new scars [8]. IntraleSIONal injections are often accompanied by adverse effects and fail to maintain a long-lasting high local concentration at the injection site [9, 10]. Topical drug application has the advantages of high specificity, convenience and few side effects but is less effective [11]. Compared to normal skin, HS has a thicker dermis with denser collagen fibers [12]. Therefore, enhancing transdermal efficiency is a key factor in the treatment of HS.

Based on dot-matrix photothermalysis theory, ablative fractional lasers (AFLs) have been proven to be able to increase the dermal absorption of topically administered drugs [13, 14] by disrupting the stratum corneum temporarily [15, 16]. AFLs have been extensively used to increase clinical efficacy in the treatment of skin diseases such as nonmelanoma skin cancer, vitiligo, melasma, scarring and alopecia [17–20]. As an AFL, a CO2 fractional laser has been confirmed to improve the elasticity, thickness, appearance and symptoms of mature hypertrophic burn scars [21]. However, most research currently focuses on in vitro experiments without long-term observation. Therefore, dynamic drug distribution over time has not been observed [22–24]. Additionally, due to rapid re-epithelialization [25], short drug penetration time intervals limit the use of CO2 fractional laser-assisted drug delivery [15, 26]. As such, the application of CO2 fractional laser-assisted drug delivery for the treatment of scars deserves more research attention with regard to enhancing the penetration of drugs.

In recent years, multiple nanocarriers with transdermal capacities have been developed and applied in AFL-assisted drug delivery, improving the therapeutic effect of drugs on HSs [27]. Silk nanofiber hydrogel (SNF) is a type of natural nanofiber with a high biocompatibility, good water dispersibility, tailorable biodegradability, low bacterial attachment and good mechanical characteristics [28]. The SNF structure is characterized by an abundance of β-sheet structures, which enhance its mechanical properties. Moreover, SNFs contain various functional groups, such as amines, phenol and alcohol, which make it possible to incorporate various drugs within or on the surface of SNFs. In addition, SNFs enhance drug concentrations in lesions as drugs are delivered at a sustained rate [29, 30]. Thus, SNFs have been introduced as powerful drug carriers that can be applied in different biomedical fields [31, 32]. It is anticipated that drug-laden SNFs cover the HSs treated by CO2 fractional lasers to achieve better transdermal delivery.

In this study, we aimed to reveal whether CO2 fractional laser-mediated drug-laden SNFs could penetrate HSs more effectively. Water-soluble rhodamine 6G (R6G) and water-insoluble rhodamine 110 (R110) as hydrophilic and hydrophobic drug models were loaded on SNFs. When treatment with a CO2 fractional laser was applied in a rabbit HS model, the drug-laden SNFs were applied to the HSs to evaluate transdermal penetration in vivo. Additionally, the week-long in vivo experiment provided a link between the healing of laser channels and drug penetration for future studies.

Methods

Preparation of SNFs

The original silk fibroin solution (6 wt%) was prepared through previously reported procedures [32]. It was concentrated to ~20 wt% over 24 h at 60°C to form metastable nanoparticles and then diluted to 2 wt% with deionized water. This diluted silk solution was incubated at 60°C in a constant-temperature drying oven for >24 h until hydrogels formed. The SNF preparation was conducted by the National Engineering Laboratory for Modern Silk & Collaborative Innovation Center of Suzhou Nano Science and Technology (Soochow University, Suzhou 215 123, People’s Republic of China).

Preparation of 0.03% R6G@SNF and 0.03% R110@SNF

Hydrophobic and hydrophilic drugs were loaded on SNFs via various processes. For hydrophilic R6G (Acros Organics), the drug was added to the SNF solution directly and then loaded on the nanofibers after stirring for 4 h at room temperature. Hydrophobic drugs were loaded on the SNF through a blending–centrifuging process. R110 (Shanghai Yien Chemical Technology Co. Ltd) was dissolved in ethanol and then blended with SNF aqueous solution. The blend solution was stirred for 24 h at room temperature to induce the transfer of R110 from ethanol to the SNF. Then, the blend solution was centrifuged at 10000 rpm for 20 min to separate the R110-laden SNF from ethanol. The specific steps were as follows. R6G (3 mg) was dissolved in 100 µl of deionized water and the solution was added to 10 ml of 2 wt% SNF, immediately followed by 4 h of stirring using magnetic stir bars at room temperature. R110 (18 mg) was dissolved in 1 ml of 100% ethanol and mixed completely. The solution was slowly added to 20 ml of 2 wt% SNF and swirled for 24 h at room temperature. Then, the mixture was transferred to a centrifuge tube and subsequently centrifuged at 10000 rcf for 20 min to obtain molecule-loaded nanofibers. The supernatant was collected after washing with deionized water three times for the quantitative analysis of the unloaded R110 content using a UV–vis spectrophotometer (Cary5000, Agilent, Santa Clara, USA). Through optimizing the conditions, the R6G and R110 loading capacities were 0.03 and 0.18%, respectively. R110@SNF (0.18%) was diluted with 2 wt% SNF to eventually obtain 0.03% R110@SNF. For the control, we also prepared a 0.03% R6G aqueous solution and a 0.03% R110 ethanol solution. The whole process was shielded from light.
Characterization

The sample morphology was examined by atomic force microscopy (AFM, Bruker Dimension ICON, Germany). Samples were diluted to 1:1000 and dispersed in ultrasound for 10 min. Then, a 2 μl solution was spin-coated onto a clean mica surface and the surface was blown with nitrogen. A confocal Raman spectrometer (InVia Qontor, Renishaw, 785 nm diode laser) was used to evaluate the loading of R6G and R110 in the SNFs. The rheological properties of all hydrogels were measured with a rheometer (Mars40, Thermo Fisher Scientific, USA). Frequency sweeps were collected continuously over a wide frequency range from 0.1 to 100 rad s$^{-1}$ at 25°C.

Isolation and culture of human keloid fibroblasts and human dermal fibroblasts

Human keloid tissues were obtained from 18- to 45-year-old Chinese plastic surgery patients with informed consent at Shanghai Ninth People’s Hospital. To collect human dermal fibroblasts (HDFs), foreskins were obtained from Chinese circumcision patients with informed consent at Shanghai Ninth People’s Hospital. This study was performed according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by Shanghai Ninth People’s Hospital. The human tissues were rinsed in Dulbecco’s phosphate buffered saline, the epidermis tissue was cut into small pieces (1–2 cm$^2$), and subcutaneous fat was removed and subsequently soaked in cell culture medium (Gibco) containing 2% penicillin-streptomycin (Gibco) and 10% foetal bovine serum (Gibco).
for 30 min. Next, the small tissues were minced and digested in cell culture medium containing 0.2% collagenase (Collagenase NB 4 Standard Grade, Nordmark, Germany) for 2–4 h in a 37°C incubator. Afterwards, the cell suspension was filtered through a 70 μm filter and centrifuged at 1200 rpm for 5 min. Finally, the cell pellet was resuspended in cell culture medium (Gibco) supplemented with 10% foetal bovine serum (Gibco) and 1% penicillin–streptomycin (Gibco), and the cells were cultured at 37°C in 5% CO₂ with the medium changed every 3 days. For experiments, the cell passage was ≤5.

**In vitro cytocompatibility of SNFs**

Cell proliferation was evaluated with a cell counting kit 8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Human keloid fibroblasts (HKF) and HDF cells were seeded in 96-well plates with 2000 cells per well. After 24 h of incubation, they were treated with 0.5, 1, 2, 4 or 8 mg/ml SNF dissolved in cell culture medium for 1, 3, 5 or 7 days. At the corresponding time, cell culture medium containing 10% CCK-8 solution was added to each well and incubated for 1 h at 37°C. Finally, the absorbances were obtained at 450 nm using a microplate reader (Multiskan FC, Thermo Scientific).

**Rabbit ear HS model**

Two-week-old male New Zealand white rabbits weighing 2 kg (SLAC, Shanghai) were kept individually. Xylazine Hydrochloride was combined with 1.5 mg ketamine per 100 g weight of rabbits and administered intramuscularly. All of the wounds were located around the center point of the rabbit ears and were distributed equally. Each circular wound was removed with a diameter of 1 cm of full-thickness skin and perichondrium while the cartilage remained intact. The rabbit ear wounds healed and were repaired entirely, with a noticeable diameter of ∼0.9 cm of HSs after 28 days. The scars appeared as bright red bumps on the skin, with obvious hyperplasia and characteristics of being thick and hard.

**In vivo transdermal drug penetration assay in rabbit ear HS model**

Mature rabbits with hypertrophic ear scars were randomly divided into eight groups: the control groups received topical treatment with R6G, R6G@SNF, R110 or R110@SNF, and the laser irradiation groups additionally received topical CO₂ fractional laser treatment (L+R6G, L+R6G@SNF, L+R110 or L+R110@SNF). The laser was operated in DeepFX mode under the parameters of 25 MJ energy intensity, 20% coverage, 300 Hz emission frequency and on the 10th spot size without overlap. The rabbits were well anesthetized before laser irradiation via intramuscular injection anesthesia. All groups were subsequently given the corresponding drug treatments (50 μL drug/HS). After 1, 3, 5 and 7 days of treatment, rabbits were sacrificed by intravenous injection of an overdose of sodium pentobarbital and HS tissues were excised. These HS tissues were then cut into frozen sections with a 7 μm thickness. Finally, the frozen
**Figure 4.** Penetration of R6G@SNF and R6G in rabbit ear HSs in vivo. (a) Confocal fluorescence images of cross-sections of rabbit HSs with different treatments. L + R6G@SNF: laser treatment and R6G@SNF application; L + R6G: laser treatment and R6G application; R6G: R6G application only; R6G@SNF: R6G@SNF application only. Scale bar: 100 μm. (b) Fluorescence intensity (FI) mean value of CZ. Statistical comparisons were made using two-way ANOVA with Tukey’s multiple comparisons; ns, no statistical significance. Data presented as the means ± standard deviation, n=6. R6G Rhodamine 6G, R6G@SNF 0.03 wt% R6G-laden silk nanofiber hydrogel, HSs hypertrophic scars, L laser, CZ coagulation zone, ANOVA analysis of variance.

Sections were viewed under a Zeiss LSM 880 confocal microscope (Zeiss Co., Germany). The fluorescence intensity was measured by ZEN 2.6 software.

**Statistical analysis**

All error bars represent standard deviations. Data analyses were conducted using GraphPad Prism 9.0 software. When
Figure 5. Comparison of R6G@SNF and R6G penetration in rabbit ear HSs in vivo. (a, c, e, g) Fluorescence intensity (FI) curves with penetration depth. (b, d, f, h) AUC of each group. Statistical comparisons were made using one-way ANOVA with Tukey’s multiple comparisons; ns, no statistical significance, ****p ≤ 0.0001.

Data presented as the means ± standard deviation, n = 6. R6G Rhodamine 6G, R6G@SNF 0.03 wt% R6G-laden silk nanofiber hydrogel, HSs hypertrophic scars, AUC area under the fluorescence intensity curves, ANOVA analysis of variance.
Figure 6. Penetration of R110@SNF and R110 in rabbit ear HSs in vivo. (a) Confocal fluorescence images of cross-sections of rabbit HSs with different treatments. L + R110@SNF: laser treatment and R110@SNF application; L + R110: laser treatment and R110 application; R110: R110 application only; R110@SNF: R110@SNF application only. Scale bar: 100 μm. (b) Fluorescence intensity (FI) mean value of CZ. Statistical comparisons were made using two-way ANOVA with Tukey’s multiple comparisons; ns, no statistical significance. Data presented as the means ± standard deviation, n=6. R110 rhodamine 110, R110@SNF 0.03 wt% R110-laden silk nanofiber hydrogel, HSs hypertrophic scars, L laser, CZ coagulation zone, ANOVA analysis of variance.
Figure 7. Comparison of R110@SNF and R110 penetration in rabbit ear HSs in vivo. (a, c, e, g) Fluorescence intensity (FI) curves with penetration depth. (b, d, f, h) AUC of each group. Statistical comparisons were made using one-way ANOVA with Tukey’s multiple comparisons; ns no statistical significance, **p ≤ 0.01 and ***p ≤ 0.001. Data presented as the means ± standard deviation, n=6. R110 rhodamine 110, R110@SNF 0.03 wt% R110-laden silk nanofiber hydrogel, HSs hypertrophic scars, AUC area under the fluorescence intensity curves, ANOVA analysis of variance.
comparing more than two samples, either one- or two-way analysis of variance was used, with Tukey’s multiple comparisons test. *P* values < 0.05 were considered statistically significant.

**Results**

**Characterization of R6G@SNF and R110@SNF**

AFM images showed the morphology changes of SNF after drug loading. Similar to the results of a previous study [31], the loaded R6G and R110 induced slight increases in SNF diameter without significant aggregation, indicating homogeneous drug distributions (Figure 1a, b, c). In addition to the typical peaks of SNF at ~1666 cm⁻¹, the Raman spectrum of R6G@SNF exhibited the typical peaks of R6G at ~1367 and 1515 cm⁻¹, while that of R110@SNF showed the typical peaks of R110 at ~1368 and 1506 cm⁻¹ (Figure 1e, f). The results confirmed the successful loading of drugs on the SNFs. After the introduction of R6G and R110, the SNFs maintained their shear-thinning capacity, similar to drug-free SNF (Figure 1g). The physical appearance of SNF, R6G@SNF and R110@SNF are shown in Figure 1d.

**Cytocompatibility of SNF**

Previous studies have revealed that SNF, which is derived from natural protein, had an antioxidant capacity and could attenuate the toxicity of the loaded drugs [31]. The effects of SNF on HDF and HKF cell viability were assessed by CCK-8 assays. When cultured for 1, 3, 5 and 7 days, both the HDF and HKF cells cultured with SNFs at different concentrations exhibited excellent viability (Figure 2a, b). These results suggested the excellent cytocompatibility of SNF. SNF as a natural compatible carrier is a preferred option for *in vivo* experiments.

**Penetration of R6G@SNF and R6G in rabbit ear HSs in vivo**

The successful rabbit ear HS model is shown in Figure 3b. R6G as a hydrophilic drug model could be visualized directly through fluorescence. To reveal the penetration of the water-soluble drugs in the laser channels, the fluorescence intensity at the penetration depth in the peri-coagulation zone (peri-CZ) (the tissue around the CZ) and the intensity mean value in the CZ were measured separately (Figure 3a). As for the control groups without laser treatment, we only measured the fluorescence intensity at the penetration depth. We found that the red fluorescence was more concentrated in the epidermis and dermis for the laser-treated groups (Figure 4a), while the red fluorescence was concentrated only in the epidermis for the groups without laser irradiation (Figure 4b). According to the results of the fluorescence quantitative curve (Figure 5a, c, e, g) and the area under the curve (representing the total penetration of drugs in the peri-CZ, Figure 5b, d, f, h), L + R6G@SNF had more penetration through the laser channels compared to L + R6G on day 1 (Figure 5a, b; day 1 L + R6G@SNF: 5987 ± 900 vs day 1 L + R6G: 3941 ± 919.8, in 6 HSs, *p* < 0.0001). This indicated that R6G@SNF could significantly enhance the penetration of R6G with the beneficial application of a CO₂ fractional laser within 24 h. However, the effect of SNF was less effective in the following days. For the groups without laser treatment, the dermis was almost completely impermeable to R6G@SNF and R6G, and no significant difference was found between the R6G@SNF and R6G groups (Figure 5). Additionally, for the laser treatment groups, the mean fluorescence intensity of the CZ was high and virtually unchanged on days 1, 3 and 5 (Figure 4b; all wounds lost their residual CZ on day 7), signifying a sustained high-drug-concentration in the CZ. Overall, with the aid of laser irradiation, R6G@SNF could effectively enhance transdermal delivery in the rabbit ear HS model.

**Discussion**

This study characterized the distribution of SNFs loaded with R6G and R110 as hydrophilic and hydrophobic model drugs based on CO₂ fractional laser treatment. Researchers divided the zone irradiated by the laser beam into two parts: one was the microscopic ablation zone (MAZ), which was the zone ablated by the laser showing an ‘empty’ zone of the laser channel, and the other was the CZ, a rim of thermally coagulated tissue of variable thickness surrounding the individual microchannels, which reflected residual thermal damage [33–35]. Many earlier studies did not separately consider the permeation of the CZ and peri-CZ, whereas we respectively analyzed the fluorescence intensities of the CZ and peri-CZ as the CZ was an inactivated tissue. The current study was conducted *in vivo* in a rabbit ear HS model.
enhanced by CO2 fractional laser irradiation to enhance. Similarly, Olesen et al. argued that a CZ of an appropriate thickness could serve as a reservoir for a small hydrophilic drug, promoting drug diffusion into the surrounding skin [35]. Research about CZ thickness found that the uptake of topical compounds was higher through microchannels surrounded by a CZ than for those without a CZ. Moreover, the highest skin uptake of compounds was observed for a CZ of 20 µm, compared to that of CZs of 80 and 0 µm [43]. In our study, the CZ thickness was ~25 µm (data not shown), which was a suitable thickness for promoting penetration. It is noteworthy that the observation time window of these studies was limited to a few hours in vitro. However, in this in vivo study, we found that for a long time, drugs could not permeate through the CZ. We speculated that rapid re-epithelialization of the CZ may be the key to unsustainable drug release. Another potential reason might be that the function of vascular/lymphatic systems and metabolism is to contribute to drug elimination [35].

Recently, a randomized clinical trial found that no significant long-term difference in scar flattening was observed between a laser + corticosteroid group and the control group [44]. However, another clinical trial reached a different conclusion: that combining CO2 fractional laser irradiation and topical 5-FU or verapamil hydrochloride treatment resulted in a significant scar treatment effect compared to the control group [45]. We speculated the main reason for these different results was that 5-FU and verapamil hydrochloride are hydrophilic while corticosteroid is hydrophobic, which validates our results.

The treatment of abnormal scar tissue remains a great challenge in clinical medicine since few carriers can penetrate scar tissue to enhance the biological function of the loaded drugs. Developing a transdermal carrier that can be loaded with drugs with distinct properties and penetrate scar tissue...
is critical for improving the efficacy of abnormal scar treatment [26]. SNF could be loaded with and immobilize both hydrophobic and hydrophilic cargos and achieve controllable and sustained release [31]. Further research indicated that SNF could penetrate the corneum layer and cell membrane by tuning its size and shape, indicating its possible applications in scar treatment [37, 38, 46]. To verify the feasibility of SNF as a transdermal carrier in scar tissue, rhodamine, a biological dye, was loaded on SNF to visualize the SNF distribution in the scar tissue. Since the permeability of SNF in scar tissue has been proved, in a future study bioactive drugs will be loaded on carriers to induce scar changes and the results will be evaluated. In conclusion, we demonstrated a novel method of topical drug delivery. Our results suggested that CO2 fractional laser-assisted drug delivery is more suitable for the delivery of hydrophilic drugs. In addition, SNF could serve as a penetration enhancer for hydrophilic drugs.

Conclusions
In summary, this study explored CO2 fractional laser-assisted drug-laden SNF delivery in a rabbit ear HS model. Our data indicated that the laser treatment group R6G@SNF exhibited better penetration in the peri-CZ compared to R6G on day 1, while R110@SNF exhibited less penetration in the peri-CZ. The mean fluorescence intensity of the CZ was dramatically high and virtually unchanged on days 1, 3 and 5 for the laser-treatment groups. For the groups without laser treatment, this fluorescence intensity was weak. We concluded that combining CO2 fractional laser irradiation with SNF drug delivery could improve the efficiency of R6G (hydrophilic drug) delivery within 1 day (before total re-epithelialization). In contrast, the penetration of R110 (hydrophobic drug) was limited in the peri-CZ. A major problem that hindered the long-time permeability of the drugs was rapid re-epithelialization. The major contribution of this study was combining CO2 fractional laser irradiation with SNF drug delivery to improve the efficiency of hydrophilic drug delivery, offering a novel idea for the topical administration of water-soluble drugs. To our knowledge, this is the first time that SNF and CO2 fractional laser irradiation have been combined to promote drug penetration.

Abbreviations
AFLs: ablative fractional lasers; AFM: atomic force microscopy; CCK-8: cell counting kit 8; CLSM: confocal laser scanning microscopy; CZ: coagulation zone; 5-FU: 5-fluorouracil; HDF: human dermal fibroblasts; HKF: human keloid fibroblasts; HS: hypertrophic scar; MAZ: microscopic ablation zone; peri-CZ: peri-coagulation zone; R110: rhodamine 110; R6G: rhodamine 6G; SNF: silk nanofiber hydrogel.

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Data availability
The dataset(s) supporting the conclusions of this article may be obtained from the corresponding author.

Authors’ contributions
ZZ and QL conceived and planned the experiments. YY and LL carried out the experiments and YY wrote the manuscript.

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki and approved by Ethics Committee of Shanghai Ninth People’s Hospital, Shanghai JiaoTong University School of Medicine (protocol code: SH9H-2019-TK206–1; date of approval: 02/15/2019).

Conflicts of interest
There were no commercial interests between any of the members of the working group during the compilation process. Each member of the working group made a statement regarding his/her conflict of interest.

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