



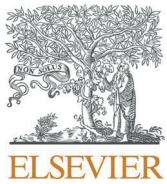
Innovation for the skin

An abstract background graphic consisting of a series of overlapping, wavy lines in a light blue color. These lines form a grid-like pattern that creates a sense of depth and movement, resembling a stylized landscape or a topographical map.

**Skin microincision technique
to enhance drug penetration for the treatment
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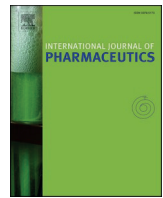


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Skin microincision technique to enhance drug penetration for the treatment of keloid and hypertrophic scars

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ABSTRACT

The synergistic effect of corticosteroids and 5-fluorouracil (5-FU) for the treatment of pathological scarring is widely documented. While topical administration can be a painless, convenient way to convey the two active ingredients, physical enhancement techniques such as microneedling are required to deepen their skin penetration and achieve the therapeutic effect. A novel approach to keloid and scar treatment is given by microincision, *i.e.*, micrometric-sized columnar perforations which allow the drugs to diffuse into the skin and promote tissue proliferation in a more physiological structure. Combining the delivery of triamcinolone acetonide (TAC) and 5-FU with microincision is an innovative approach that could improve the speed and efficacy of regenerative treatments.

This study evaluated the effectiveness of the skin treatment with a device combining microincisions and photobiomodulation, in the skin permeation of a combination of TAC and 5-FU. Increasing treatment times (4, 6, and 8 min) led to higher drug penetration compared to intact skin, with a more noticeable effect for 5-FU compared to TAC. Specifically, all treatment durations were significantly more effective ($p < 0.05$) than the control for 5-FU, while TAC showed less variation between treatments. Moreover, it was shown that *in-vitro*, the permeation improvement given by the red-light treatment was mainly due to the mechanical massage, which pushed the actives into the microchannels created by the treatment. The application of prolonged skin microincision times ensured much higher skin permeation of both TAC and 5-FU compared to microneedling on healthy excised skin.

1. Introduction

Pathological scarring affects millions of people in developed countries and represents a huge healthcare burden (Sen, 2023). Abnormalities of scarring, namely keloid and hypertrophic scars, can negatively impact patients' quality of life. Although they are not life-threatening themselves, they can affect the functionality of the body area involved and cause aesthetic imperfections with psychological consequences, as well as subjective symptoms such as pain and itching. This fairly common clinical problem is not an easy one to solve due to the complexity of the pathogenetic mechanisms, which are still not well understood, and has a high frequency of recurrences.

The physiological process of wound healing is continuous and involves several stages: inflammation, proliferation and remodeling (Frech et al., 2023). The first phase begins immediately after tissue injury, whether surgical or traumatic, and involves transient vasoconstriction, followed by platelet degranulation, activation of the

complement cascade and coagulation. Platelets and neutrophils release growth factors for keratinocytes and fibroblasts (Darby and Desmoulière, 2020; Tredget et al., 1997). In the second proliferative phase, angiogenesis, proliferation of fibroblasts, collagen III, elastin, proteoglycans, and glycosaminoglycans prevail. The final stage of remodeling involves the slow replacement of type III collagen into type I collagen (Borthwick et al., 2013). When the synthesis and degradation of collagen are unbalanced, in particular when there is an excess of inflammatory mediators leading to an increased proliferation of fibroblasts and their differentiation into myofibroblasts, the normal scar is replaced by a pathological fibrous tissue rich in type I and III collagen, fibronectin and laminin and deficient in hyaluronic acid and decorin (Lee and Jang, 2018; Scott et al., 1996; Zhang et al., 2007).

Considering the complexity of the scarring mechanism, there are several therapies for the treatment of pathological scars that follow two main lines of approach: invasive and non-invasive therapies. On the one side, invasive therapies include: a) surgical excision (Siotos et al., 2019);

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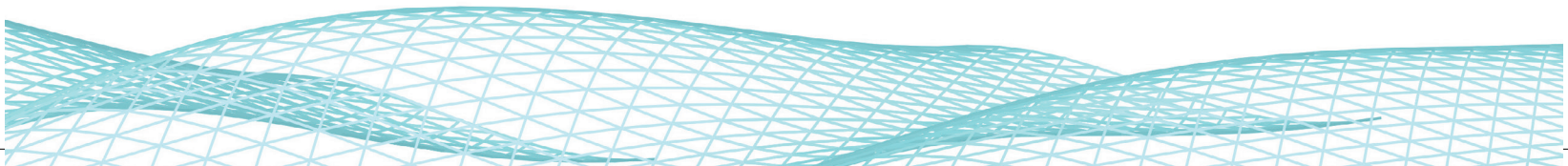
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b) fractional ablative lasers, such as CO₂ laser (Lee et al., 2016; Makboul et al., 2014; Siotos et al., 2019); c) radiotherapy (Ji et al., 2015; Miles et al., 2021); d) cryotherapy (Zouboulis, 1993); e) intralesional injections of corticosteroids, interferons and other chemotherapeutic agents (Larrabee et al., 1990; Ledon et al., 2013; Manuskiatti, 2002). On the other side, non-invasive techniques used as both prophylaxis and therapy include: a) occlusive dressings such as silicone gels (Oliveira and Gold, 2020; Perkins et al., 1983); b) compression therapy (Renò, 2002; Renò et al., 2001); c) topical treatments with vitamin E, topical retinoids, corticosteroids and 5-fluorouracil (5-FU), verapamil, imiquimod, and onion extracts (Frech et al., 2023; Gianatasio et al., 2021; Palmieri et al., 1995).

Although numerous therapies are available for the treatment of pathological scars, the evaluation of the efficacy of these therapies is still challenging, due to the lack of well-controlled clinical trials. Furthermore, the lack of predictability of individual outcomes between treatment modalities has led international groups to produce guidelines in which the combination of several therapies is suggested (Kim et al., 2013; Monstrey et al., 2014; Mustoe et al., 2002). Thus, in order to achieve better results, two or more treatment techniques are often combined, and several drugs synergistically are used, considering many characteristics, including the patient's risk factors, scar properties (keloid vs hypertrophic scar) and anatomical location (Boutli-Kasapidou et al., 2005; Monstrey et al., 2014).

In this context, the synergy between corticosteroids and 5-FU, administered by intralesional injections (Darougheh et al., 2009), is widely acknowledged in the literature and used in the medical field (Huang et al., 2013). To overcome the limitations associated with injection, a possible alternative could be cutaneous administration (Sheng et al., 2023). However, the ability of these compounds to penetrate the scar, or more in general the skin, can represent a limitation to overcome (Lin et al., 2019; Petrilli et al., 2018; Prince et al., 2018). One of the possible physical approaches to enhance drug penetration is based on breaking the stratum corneum by microincisions that should be deep enough to quickly promote the passage of molecules, but superficial enough not to come into contact with nerve fibers and blood vessels, which are located in the dermal layer (Donnelly, Singh, and Woolfson 2010). Being this skin penetration enhancement technique poorly investigated, the present work aims to provide information on the skin penetration of a fixed combination of triamcinolone acetone (TAC) and 5-FU in combination with microincision. Microneedles (MNs) were used as a positive control since their positive effect is extensively reported in the literature for a plethora of drugs.

2. Materials and methods

2.1. Materials

Materials: TAC and 5-FU were provided by Farmalabor (Milano, Italy), whereas hydroxyethylcellulose and phenoxyethanol were supplied by A.C.E.F. (Piacenza, Italy). Saline solution was supplied by Eurospital S.p.A. (Trieste, Italy). Rose Bengal was purchased from Thermo Fisher Scientific (Milano, Italy). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol, ethanol, orthophosphoric acid, and Nylon 0.22 µm filter, Teflon, Parafilm® were purchased from VWR Chemicals (Leuven, Belgium). Photopolymerizing resins were purchased from Formlabs (Formlabs Inc. Boston, MA, USA). All the other reagents were purchased of analytical grade. Fresh 'ultrapure' water (18.2 MΩ, Milli-Q grade) employed for the preparation of the formulation and HPLC solvents was produced with the Millipore Milli-Q purification system (MA, USA).

2.2. Equipment

Microincisions were procured through the APS device® (Nati, 2015). A schematic representation of the device's main features is reported in

the Supplementary material (Fig. S2). This device combines a microincision phase with a second stimulation phase.

Skin microincision: the microincision phase creates micro-incisions in the tissue by means of five protuberances in the handpiece, each composed of two concentric cones. The first cone keeps tension in the skin, allowing the second upper cone to repeatedly hit the skin and penetrate the epidermis layer up to 0.25 mm, without altering the basement membrane. This rhythmic movement in pulsed mode generates a single 'train' of 'pressure' waves, resulting in the induction of a multitude of micro traumas that stimulate the dermal cells to repair themselves, as well as promoting vascularisation. In addition, when an active is applied to the skin, this first skin microincision phase mechanically presses the drug into the microincisions, aiding its absorption (APS, 2023).

Second stimulation: the instrument has a corona of high-power red LEDs (630 nm) on the outside of the cup, which allows considerable biostimulation effects on fibroblasts and elastin by emitting a coherent monochromatic non-collimated light with a very small wavelength variability (±5%) (APS, 2023).

Both the skin microincision phase and the biostimulation phase were performed with circular movements on the skin surface, akin to a skin massage.

2.3. Methods

2.3.1. Preparation and characterization of gel loaded with TAC and 5-FU

A hydrogel containing 1.2 % w/w TAC and 5.0 % w/w 5-FU gel was prepared as follows. The gelling agent (1.2 % w/w hydroxyethylcellulose) was dispersed in water, and heated at 50 °C under mechanical agitation until complete dispersion. The dispersion was left to cool for 1 h to allow gel formation. Then the ethanol solution containing the two drugs and the preservative (1.0 % w/w phenoxyethanol) was added to the base gel at room temperature stirring slowly so as not to incorporate air. The ethanol/water ratio was set at 1/8 w/w.

The drug contents were determined using the method described in section 2.3.5.

The viscosity of the gel was measured by a rotational viscometer (Rotavisc me-vi Viscometer, version: 1.02.004/1.11.004, IKA, Germany). The analysis was conducted at a stable temperature of 25 ± 1 °C. The VOL-SP-7.1 spindle suitable for low viscosity semi-solids was chosen. Viscosity readings were taken for 60 s at a rotation speed of 25.00 rpm. The results were expressed as the average of three different samples.

2.3.2. Microneedles preparation and characterization

MNs arrays were prepared by stereolithography (SLA) with the 3D printer Form 3B+ (Formlabs, Germany). The arrays were designed with CAD software as 7 × 7 square-based pyramids with 200 µm diameter, 1000 µm height, and distance between the base centers of 1000 µm. The systems were 3D printed with a PMMA photopolymerizable resin using a z-axis resolution of 0.025 µm, then the excess resin was removed with a 2-propanol wash for 20 min, followed by a second UV curing (60 °C, 20 min) to improve the mechanical properties of the MNs arrays.

The geometrical features of printed MNs were observed under a stereomicroscope (Nikon SMZ745T, Nikon Europe BV). Skin penetration depth was estimated using a synthetic model composed of 8 layers of Parafilm® wrapped in a single aluminum foil (Larrañeta et al., 2014), pressing the needle with a tensile testing machine (Instron 5965, compression mode, 30 N for 30 s, speed of 1 mm/min).

2.3.3. In-vitro skin incision and drug diffusion study

Skin samples were mounted on Franz diffusion cells with the stratum corneum facing the donor compartment. The diffusion area and the volume of the receptor compartment were 0.636 cm² and 3.0–3.2 mL, respectively. The receiver compartments were filled with a degassed saline solution 0.9 % w/v (Fantini et al., 2019; Naguib et al., 2014),

previously filtered with a 0.22 µm nylon filter. Special care was given to avoid air bubbles between the membrane and the solution in the receptor compartment. The medium was maintained under stirring and at a controlled temperature of 32 ± 1 °C by means of a circulating water bath. Full-thickness porcine ear skin samples were die-cut into 25 mm diam circles and mounted onto Franz Diffusion Cells. The skin integrity was assessed by measuring the baseline electrical resistance (R_0) after an equilibration time of 15 min, using saline solution (0.5 ml) in the donor compartment, and an Agilent 4263B LCR Meter (Microlease, Italy), with voltage 100 mV and frequency 100 Hz, according to an internal procedure (Volontè et al., 2024). The skin sample was used for the permeation studies only if $R_0 > 5.0$ kΩ.

Permeation studies were performed by applying 100 µl of gel on the skin before the treatment with either microincisions or microneedling. Subsequently, a further aliquot of 50 µl gel was loaded and the second treatment was performed. The experimental conditions are summarized in Table 1. Microneedling was performed by pressing the needles (paragraph 2.3.2) on the skin surface with a force of 30 N, maintained for 60 s. The conditions of treatment F (i.e., replacing the biomodulation step with a rotational movement to mimic the handpiece movement) were introduced to better discriminate the effect of the mechanical stimulation from the effect of photostimulation, which should be ineffective in *in-vitro* skin permeation studies. As a control for skin permeation of all conditions, a total aliquot of 150 µl gel was loaded directly on a skin sample.

At predetermined time intervals over 5 h (0.5/1/3/5h), 200 µl samples were withdrawn from the receiver compartment and replaced with an equal volume of fresh medium. Sink conditions were maintained throughout the experiments. The amount of TAC and 5-FU at each time were assayed using the analytical method detailed in 2.3.5 section.

The cumulative amount of TAC and 5-FU permeated through the skin per unit area was calculated starting from the concentrations withdrawn from the receiver compartment of the Franz diffusion cell according to the Equation (1):

$$Q_t \left(\frac{\mu\text{g}}{\text{cm}^2} \right) = \frac{C_t \left(\frac{\mu\text{g}}{\text{mL}} \right) \times V(\text{mL}) + C_{t-1} \left(\frac{\mu\text{g}}{\text{mL}} \right) \times 0.2(\text{mL}) + C_{t-2} \left(\frac{\mu\text{g}}{\text{mL}} \right) \times 0.2(\text{mL}) + C_{t-n} \left(\frac{\mu\text{g}}{\text{mL}} \right) \times 0.2(\text{mL})}{0.636 \text{ cm}^2} \quad (1)$$

where Q_t is the permeated amount at the time t , C_t is the drug concentration at the same time point, V is the volume of the Franz's Cell, 0.2 ml is the volume withdrawn from the receiver compartment at each time point and 0.636 cm^2 is the surface area of the Franz cell. The Q_{P_t} values

Table 1
Skin treatment conditions.

Condition	First treatment	Second treatment
A	Microincision 2 min	red LEDs 2 min
B	Microincision 3 min	red LEDs 3 min
C	Microincision 4 min	red LEDs 4 min
D*	Microincision 2 min	–
E	Microincision 4 min	–
F	Microincision 4 min	Application of rotational movement by a Teflon lid for 4 min
G	Microneedling	–

* Treatment D was considered only for *in-vitro* stratum corneum integrity evaluation.

were plotted as a function of time (t).

Skin retention of the drugs over 5 h following application of the formulation was evaluated. At the end of the permeation time, the porcine membranes were carefully removed from Franz's Diffusion Cells and the residual gel on the skin surface was mechanically removed. Each membrane was gently washed with 10 mL of methanol to remove the unabsorbed drugs. Subsequently, the membrane was dried, thinly sliced, and collected in 4 mL of fresh methanol. The suspensions were sonicated using Ultrasonic Processor (UP200St, Hielsher, equipped with the piezoelectric probe type 2) for 15 min and then maintained at 4–6 °C for 24 h. Finally, the supernatant was filtered through a filter membrane with a pore size of 0.45 µm and assayed by HPLC. The retained TAC and 5-FU amount (Q_{r24}) were expressed as micrograms per unit area of skin membrane. The obtained results were expressed as an average of parallel experiments performed at least in triplicate.

The permeation enhancement ratios (ER) between tested conditions (A–G) versus control were calculated by exponentiating the difference of the logits between the two series, using SAS 9.4 (SAS Institute Inc., US). For each comparison, the ER was calculated in terms of $Q_{0.5}$ and J -values.

2.3.4. *In-vitro* stratum corneum barrier property evaluation

The ability of the treatment D, E or G to break the stratum corneum barrier properties was preliminarily determined by evaluating the drop of electrical resistance or by staining the skin surface with Rose Bengal.

Skin samples' electrical resistance was evaluated with Agilent 4263B LCR Meter as baseline, and recorded immediately after the treatments to verify the generation of the microincisions, and recorded over 240 min.

The staining was performed by flooding the membranes with 200 µl of a Rose Bengal solution 1 mg/ml in PBS pH 7.4, and after 60 s, the excess solution was removed. The skin samples were then gently wiped with delicate tissue (Kimwipes, Kimtech) and washed with 1 ml PBS pH 7.4. The samples were then mounted on a microscope slide and observed under a Nikon Eclipse Ts2 fluorescence microscope (Nikon, Japan), at a

$4 \times$ magnification, using an excitation bandwidth of 540/25 nm, and collecting the emitted fluorescence at 605/55 nm. The obtained micrographs were elaborated with ImageJ software with the processing package FIJI, version 2.15.0.

2.3.5. Quantification of TAC and 5-FU

The quantitative analyses of TAC and 5-FU were performed by high performance liquid chromatography (Agilent HP 1100 ChemStations, Agilent Technologies, USA) and the chromatographic data were acquired and processed by ChemStation for LC 3D software. No interfering peaks from semisolid excipients were found in both methods.

TAC. The compound separation was carried out using a C-18 reverse-phase column (InertClone™ 5 µm ODS(3), 250 mm × 4.6 mm, Phenomenex, USA) set at 25 °C (room temperature). Acetonitrile/water (55/45 v/v) was used as the mobile phase. A sample of 20 µl was injected for an isocratic elution at a flow rate of 1.0 ml/min. The wavelength for detection was set at 238 nm (Fantini et al., 2019). The retention time of TAC was 4.5 min and two calibration curves were constructed in the overall range of 0.1–100 µg/ml ($R^2 = 0.99999$).

5-FU. The compound separation was carried out using C-18 reverse-phase column (Luna® Omega 3 µm PS C18, 150 mm × 4.6 mm, Phenomenex, USA) set at 15 °C. The mobile phase was water acidified with 0.1 % orthophosphoric acid/methanol (93/7 v/v). A sample of 20 µl was

injected for an isocratic elution at a flow rate of 0.6 ml/min. The wavelength for detection was set at 265 nm (Khan and Wong, 2018). The retention time of 5-FU was 4.0 min and two calibration curves were constructed in the overall range of 0.1–100 µg/ml ($R^2 = 0.99999$).

2.4. Statistical analysis

Statistical analyses were performed using Two-way analysis of variance (ANOVA) with post-hoc comparisons (Tukey), where appropriate, with $p < 0.05$ as a level of significance (GraphPad Prism software 10.2.2 version – GraphPad Software, CA, USA).

3. Results

MNs arrays were produced as the positive control and their properties are reported in the Supplementary material (Table S1, Fig. S1).

The evaluation of the skin integrity after skin microincision or microneedling evidenced that all treatments caused a significant decrease in the electric resistance (Fig. 1A). The drop in the resistance is a representation of the barrier disruption, meaning that micro-perforations in the stratum corneum efficiently occurred in all the treatment conditions. Previous studies reported that human skin perforated with solid MNs showed a pore size reduction of ~ 25 % within the first 30 min, then a slow and steady re-sealing of the skin, which could occur in 6 h, both *ex-vivo* and *in-vivo* (Gupta et al., 2011; Haridass et al., 2019). However, in our *in-vitro* setup only a slow recovery in the resistance values was found even after 4 h regardless of the type of treatment, as shown in Fig. 1B. In particular, a rapid partial recovery of the skin electrical resistance within the first 30 min was assessed after MNs treatment, while a slower, steady recovery was registered after skin microincisions.

The ability of skin microincision or microneedling to break the stratum corneum was confirmed by visualization of the skin lesions by means of Rose Bengal (RB), which is a fluorescein derivative generally used to evaluate epithelial integrity (Fig. 2). As expected, the images denote a dependence of the number of microincisions from the treatment time (treatment D vs treatment E of Table 1). Furthermore, 30 min after treatment D, the RB staining revealed that the skin microcores were still visible (Fig. 2, panel D).

Fig. 3 shows the *in-vitro* permeation profile of TAC (Fig. 3A) and 5-FU (Fig. 3B). As expected from the data on skin integrity, all treatments were able to enhance the passive diffusion of the two compounds either considering the first point of the curve (enhancement ratio on $Q_{0.5}$ values, Fig. 3C) or the whole permeation profile (enhancement ratio on J values, Fig. 3D). Moreover, considering that the effect of the stimulation during the second phase of the APS device did not occur, in this set of *in-vitro* experiments, the increment of the enhancement factor in the case of treatments A-C was attributed to the massage of the skin performed post treatment. Indeed, the enhancement factor of treatment E (i.e., 4 min skin microincision, which was the most invasive physical treatment) was

lower than treatment C and treatment F. In general, the treatment time and the following mechanical massage associated with the second treatment phase resulted effective in forcing both drugs through the microincisions. The permeation profiles of both TAC and 5-FU after treatments C and F did not show significant differences ($p > 0.05$), thus confirming that the enhancement effect given by the second treatment was mainly given by the “massage” which further pushed the pharmaceutical form into the created microchannels. This *in-vitro* result corroborates other studies (Chen et al., 2017; Walsh et al., 2023) showing how light improves the outcome in scars treatment when combined with corticosteroids and 5-FU: although the effect of red-light has not been observed *in-vitro*, mechanical massage alone in the second treatment step also increased the permeation profile, suggesting the positive and synergistic effect of the two treatment steps performed with devices combining micropunctations and photobiomodulation.

However, it should be underlined that the effect of microincisions or microneedling resulted more effective for 5-FU than TAC. This different pattern was attributed to the higher hydrophilicity of 5-FU ($\log P = -0.9$) with respect to TAC ($\log P = 1.2$). Indeed, the breaking of the stratum corneum led to a preferential diffusion of 5-FU through the hydrophilic holes opened by microneedling or microinciding.

This phenomenon was attributed to the higher hydrophilicity of 5-FU which can diffuse through the hydrophilic channels derived by the breaking of the stratum corneum. The positive effect of this type of physical enhancement treatment was already described for 5-FU. Indeed, the pretreatment of the skin with metal-based MNs (Derma-roller® with 192 needles of 500 µm in length, 50 µm in base diameter) significantly increased the *in-vivo* antitumor activity of 5-FU topically applied (Naguib et al., 2014). In contrast, TAC itself has higher lipophilicity; therefore, TAC shows more affinity towards the outmost layer (stratum corneum) and is less able to exploit hydrophilic channels to diffuse into the skin, requiring direct intradermal delivery to reach the deepest skin strata. For this reason, the enhancement effect of microincisions resulted significantly different only when the treatment time increased up to 4 min (treatments C and F), which deeply affected the integrity of the stratum corneum and pushed higher TAC amounts in the skin.

As expected, the effect of the two types of treatments on skin retention resulted less evident for both the drugs (Table 2).

4. Discussion

For decades, physicians have turned to a variety of techniques to treat pathological scars, including the delivery of TAC and 5-FU, alone or in combination. To achieve this aim, marketed ointments and creams containing these drugs have been historically applied to improve the general poor bioavailability of both drugs (Cohen and Stoughton, 1974; Goodman and Barry, 1988; Sigg and Daniels, 2021). More effective results can be obtained using intralesional injections, which are currently the primary choice in treating pathological scars, although the outcome

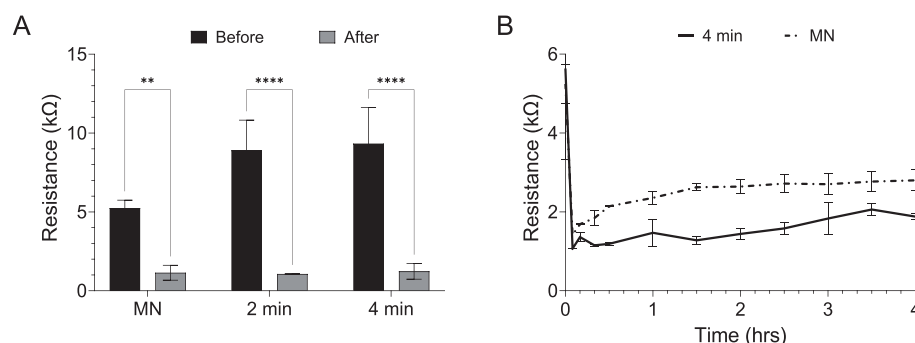


Fig. 1. A) Skin electrical resistance values before (black) and after (grey) MNs treatment (MN) or microincision treatment (under conditions D and E). Values are expressed as mean $\pm$ standard deviation, std ($n = 3$). B) Skin electrical resistance values over time after 4 min microincision, treatment C ($n = 3$, mean $\pm$ std).

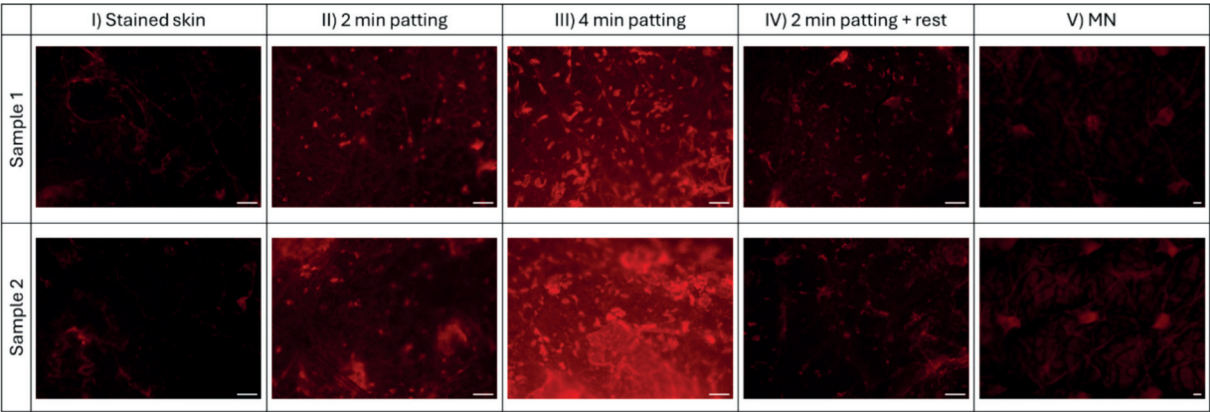


Fig. 2. Fluorescence images of RB-stained skin without physical treatment (panel I), immediately after treatment D (panel II), treatment E (panel III), and treatment G (panel V). Panel IV shows the staining performed after 30 min rest following the treatment D. Scalebar: 200 μm .

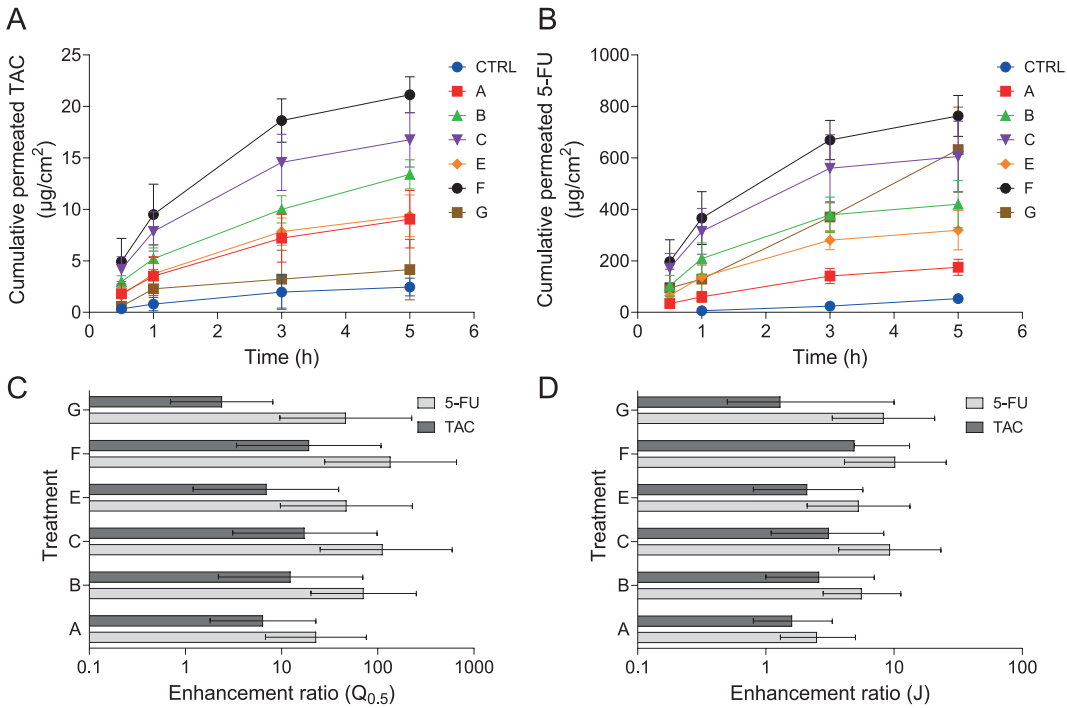


Fig. 3. Skin permeation profiles of TAC (panel A) and 5-FU (panel B) over 5 hrs of application, after skin treatments A-C and E-G. Data are expressed as mean $\pm$ std ($n \geq 3$). Panels C and D show the enhancement ratio (ER) of TAC and 5-FU of tested conditions versus control. The results are expressed as ER arithmetic means (90 % confidence intervals) of $Q_{0.5}$ and J-values, compared to the permeation data from intact skin. All the values are significantly different with respect to the reference ($p < 0.05$ as a level of the significance of tested condition versus control obtained by Dunnett post-hoc test).

Table 2
Skin retention amounts after the different treatments. Data are expressed as mean concentration ($\mu\text{g}/\text{cm}^2$) and standard deviation (std).

Drug	Condition						
	CTRL	A	B	C	E	F	G
5-FU	10.6 ± 4.6	13.7 ± 4.2	44.9 $\pm$ 26.7	28.5 $\pm$ 10.0	19.6 ± 6.2	44.9 $\pm$ 26.8	47.1 $\pm$ 17.5
TAC	1.4 $\pm$ 0.9	3.2 $\pm$ 0.6	2.2 $\pm$ 1.4	1.4 $\pm$ 0.3	4.5 $\pm$ 1.5	1.0 $\pm$ 0.2	1.3 $\pm$ 0.7

and the lack of side effects are heavily dependent on the skills of the medical professionals administering the injection (Sheng et al., 2023). To improve local application and reduce systemic drawbacks, alternative strategies have emerged, such as occlusion, to alter the skin barrier,

or microneedling, to completely bypass the stratum corneum barrier. The data obtained in this work suggest that the use of physical enhancement methods able to break the stratum corneum significantly improves the skin penetration of this drug combination, avoiding the risks related to the local intradermal administration. In particular, this approach resulted particularly efficient when microincisions were applied for 4 min. Indeed, the increase of the fluxes of TAC and 5-FU resulted 4 times and double, respectively, with respect to the effect of microneedling. Moreover, the enhancement effect obtained with occlusion, that is the enhancement approach of some topical formulations of TAC and 5-FU currently available, especially as galenic preparations, did not lead to the same results and consequently, the bioavailability in pathological tissues is lower and can be insufficient to achieve a complete remission (David and Lowe, 1989; Toffoli et al., 2023).

An example of a more invasive physical skin penetration

enhancement technique is given by microneedling. MNs are widely studied systems that mechanically disrupt the skin by creating transient microchannels, thus completely overcoming the barrier given by stratum corneum and favoring the diffusion of a wide variety of drugs. For this reason, MNs arrays were proven to be a convenient delivery device, and, being less invasive than hypodermic needles, they offer the potential for increasing the patients' compliance in the treatment of pathological scars (Mbituyimana et al., 2023). Moreover, this technique *per se* can be applied for the treatment and correction of inactive keloids and hypertrophic scars by mechanically destroying the scar tissue fibers and favoring the correct regeneration of the skin. The combination of MNs and 5-FU has been proposed in the literature, showing high permeation through murine skin (Naguib et al., 2014), rabbit skin (Zhang et al., 2023), and even in a clinical trial (Costa et al., 2019), where MNs and 5-FU led to excellent or very good clinical improvement of *striae albae*. Corticosteroids levels in third-degree burns were significantly higher when the tissue underwent microneedling, but only after at least 2 hrs of diffusion (Barati et al., 2024). Several studies confirmed *in-vivo* higher bioavailability of corticosteroids loaded in dissolving MNs, as well as the efficacy and safety of the proposed combination (Chen et al., 2023; Ohn et al., 2021; Xu et al., 2025). Only a single study proposed a combination of TAC and 5-FU in dissolving MNs, as a valid alternative for the treatment of hypertrophic scars (Yang et al., 2021). Our findings on the combination of microneedling and TAC and 5-FU were in accordance with the studies previously mentioned, underlying the reliability of this approach as the positive control for this investigation.

Focusing the attention on the effect of skin ablation with the two different approaches, *i.e.* microneedling and microincision, on the whole penetration pattern of the combined drugs, the following considerations can be withdrawn. The microneedling has a positive effect on both skin permeation and skin retention, but the effect is more significant for 5-FU than TAC. This significantly affects the ratio between the amounts of the two drugs recovered in the skin (5-FU/TAC ratio) at the end of the experiments (hereinafter drug retained ratio, DRR). Indeed, the DRR value shifted from about 7 for the untreated skin (condition CTRL) to about 35 (condition G). In the case of microincision, the 5-FU:TAC permeated ratio and DRR value can be tuned by adjusting the treatment time, resulting in the longer the time, the larger the extent. This is particularly relevant in the case of fixed combinations of drugs since the tuning of the experimental conditions can modulate the penetration pattern of each drug. In particular, the condition E allowed a significant increase of 5-FU and TAC concentrations in the skin with respect to the control and condition A, which assured a DRR equal to 4.2, that is close to that used in the intralesional administration by injection (*i.e.*, 5 % 5-FU and 1.2 % TAC).

5. Conclusion

This study highlighted how skin microincision modulates the skin integrity and permeation profile of the 5-FU/TAC fixed combination of drugs according to the intensity of the treatment. Indeed, the microincision treatment alone significantly improved the permeation of both drugs, while the addition of the second treatment ensures the penetration of the active ingredients into the skin itself, as demonstrated in treatment F by substituting this step with a mechanical rubbing. All together the presented information suggested that this approach can be considered a valid and versatile physical technique to improve the skin penetration pattern of drugs.

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CRedit authorship contribution statement

Elisa Vettorato: Investigation. Paola Volonté: Investigation. Umberto M. Musazzi: Data curation. Francesco Cilurzo: Supervision. Antonella Casiraghi: Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2025.125259>.

Data availability

The data supporting the findings of the article is available at: <https://mega.nz/folder/NqVEBQAZ> upon decryption key request to the corresponding author.

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