Methotrexate Cutaneous Delivery by Fractional CO2 Laser Against 5-Fluorouracil in Stable, Nonsegmental Vitiligo

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BACKGROUND Being chronic, combination therapies in vitiligo are beneficial, especially in refractory cases. Fractional CO2 laser (FCL) delivery of 5-fluorouracil (5-FU) has been successful in stable vitiligo. Topical methotrexate (MTX) is a potential, safer alternative to systemic route in vitiligo.

OBJECTIVE To investigate efficacy/safety of FCL with MTX versus 5-FU in stable vitiligo, and to assess serum and lesional interleukin (IL)-23 as possible therapeutic markers.

MATERIALS AND METHODS A total of 42 patients with vitiligo were enrolled. FCL was applied at 50 and 100 mJ energies, followed by topical MTX in group I and 5-FU in group II for 3-monthly sessions. Modified Vitiligo Area Scoring Index (VASI) and photography evaluated response, along with evaluation of serum and lesional interleukin-23 (IL-23).

RESULTS Both MTX and 5-FU with FCL produced significant reductions, without difference, in VASI. All groups exhibited considerable repigmentation by photography, with earlier response in higher energy FCL+5-FU after treatment and continuous improvement in FCL + MTX at follow-up. Post-treatment reductions in serum and lesional IL-23 occurred in all groups, with greater decrease in serum IL-23 with MTX and lesional IL-23 with higher-energy FCL.

CONCLUSION MTX provides comparable efficacy and safety with 5-FU after FCL for stable, nonsegmental vitiligo. IL-23 is a new treatment biomarker in vitiligo.

Vitiligo causes acquired pigment loss, which significantly affects patients' life quality.[1] Current treatments include medical, surgical, and physical therapies, but none are completely satisfactory. Consequently, there is a great need to develop new combination therapies.[2]

Transdermal drug delivery implies physical techniques such as dermabrasion, microneedling, and lasers that enhance absorption and efficacy of topical drugs such as triamcinolone, 5-fluorouracil (5-FU), and methotrexate (MTX). This avoids poor bioavailability and risk of systemic adverse effects associated with oral drugs.[3,4] Fractional CO2 laser (FCL) improves vitiligo either alone or with other therapies by improving drug and ultraviolet penetration, stimulating melanoblast activity and cytokine modulation, including increased TGFβ1 and MMP9.[5] FCL was found to lower circulating interleukin-23 (IL-23) in patients with vitiligo after treatment.[6] Lesional IL-23 has not been previously studied in vitiligo.

Several studies reported satisfactory results of topical 5-FU combined with other modalities, including narrow-band ultraviolet B (NB-UVB), microneedling, and laser ablation in treating vitiligo.[7,8] Topical MTX has been recently studied in vitiligo.[9,10] Unlike oral route, topical MTX is believed not to have significant hepatotoxic and hematologic adverse effects.[10] As MTX is a large hydrophilic molecule that cannot penetrate intact skin, various drug delivery systems as nano-vehicle preparations and laser-assisted delivery, are being developed.[11] One study found that fractional Erbium laser delivery of topical MTX is effective in porcine skin, facilitating MTX distribution and concentration in mid dermis. It suggested that laser-assisted topical MTX could be an appropriate alternative to systemic MTX for some skin disorders.[12] No previous studies in literature have evaluated FCL combined with MTX in vitiligo.

This study aimed to evaluate efficacy and safety of FCL-assisted cutaneous delivery of MTX against 5-FU solutions in stable, nonsegmental vitiligo using two laser energies on clinical and immunologic levels.

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Data is available on reasonable request to the corresponding author.

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Materials and Methods

This is a randomized clinical trial; study protocol was registered at ClinicalTrials.gov with ID: NCT05008887 and obtained institutional ethical approval (IRB 17200635). Patients were randomized using a computer-generated simple randomization sequence; investigators were aware of group assignments. After writing informed consent, the study included 42 patients with vitiligo of both sexes, aged ≥18 years, who were recruited from outpatient clinics of Dermatology Department. All patients had at least 2 patches of stable vitiligo for ≥6 months, refractory to conventional therapies, and had stopped any topical medications for 1 month, phototherapy, or systemic vitiligo treatment for 3 months before enrollment. Patients with histories of skin cancers, keloids, hypertrophic scars or photosensitivity, systemic diseases, active inflammation or infection, current pregnancy/lactation, and extensive disease were excluded. In each patient, 2 symmetrical patches of comparable size and site were enrolled. In only 5 patients, 4 patches were treated. Overall, 94 patches were included in final analysis.

After detailed history and examination for all participants, laboratory investigations, including complete blood count and liver and renal chemistry, were performed before starting treatment and monthly during treatment. This was to detect possible side effects from systemic absorption of topical MTX and 5-FU.

The patients were randomly divided into 2 main treatment groups (21 patients each). Two vitiligo patches/duplicates were treated in every patient with FCL at 2 different energies (lower energy, 50 mJ and higher energy, 100 mJ), followed by topical MTX solution in group I (46 patches) and topical 5-FU in group II (48 patches) for 3-monthly sessions. FCL was performed after topical anesthesia and sterilization. Other laser parameters were pulse duration of 2 ms (with 50-mJ energy) and 5 ms (with 100-mJ energy), density of 144 spots/cm^2, and depth level 1. One to two passes with minimal overlap were used over each patch and 2 mm of perilesional skin, with erythema being the desirable end point without bleeding.

In Group I, MTX solution 1% concentration (50 mg/5 mL) was applied after laser using insulin syringe (0.1 mL, 1 cm intervals, maximum volume; 2 mL per session) followed by gentle massage. Ten minutes after, an occlusive dressing was applied for 24 hours. In Group II, 5-FU solution 5% concentration (250 mg/5 mL) was used in same way. After procedure, topical gentamicin and emollient creams were applied twice-daily for 1 week. Patients were requested not to remove crusts, to avoid trauma at treated areas, and to apply sunscreen for lesions on sun-exposed sites.

Primary outcome measures were percentage reduction in modified VASI and repigmentation grades by photographic evaluation. Secondary outcomes included changes in serum IL-23 levels, and lesional IL-23 expression, patient satisfaction, and treatment adverse events.

Patients were clinically evaluated before every session, 1 month, and 3 months (follow-up) after final treatment. For each lesion, modified VASI score was determined by product of vitiligo area in hand units (set at 1% per unit) multiplied by extent of depigmentation within each measured patch.[13] High-resolution digital photographs were additionally taken using same camera settings, lighting, and positioning (iPhone 13, a dual 12-megapixel camera). Two nontreating dermatologists then blindly reviewed photographs to assess repigmentation response by quartile grading scale,[14] interobserver agreement was evaluated. All patients were asked, 1 month after treatment, to rate overall satisfaction of their response (not satisfied: <25%, slightly satisfied: 25% to <50%, satisfied: 50% to <75%, or very satisfied: >75%).[15] Patients were also asked to report adverse events from treatment, for example, erythema, pain, infection, hyperpigmentation, scarring, or allergic reaction.

For serologic assessment of IL-23, a 3 mL whole-blood sample was collected from 40 participants (20 per group) at baseline and 1 month after last session. Serum was separated and stored at −20°C until analysis. IL-23 levels were determined using a commercial ELISA kit (Cat. No. ELK1570; ELK Biotechnology Co., Ltd, China) following manufacturer's protocol, after diluting serum samples 1:100 with the provided diluent buffer. Absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, Vermont), and IL-23 concentrations were derived from standard curve.

Immunohistochemical IL-23 evaluation was performed in 23 patients (12 in group I and 11 in group II) through punch biopsies (3 mm) from lesional skin of different treated patches, before treatment, and 1 month after last session. Histopathologic evaluation using hematoxylin and eosin stain was performed, followed by immunohistochemical evaluation using anti-IL-23 antibodies. Primary IL-23 antibody of Chongqing Biospes Co., Ltd, Chongqing, China (Catalog no. YPA2762) at a concentration of 1/100 was applied for tissue sections and incubated for 1 hour at room temperature. IL-23 immunoreactivity score was calculated as (intensity×percentage of immunoreactive cells) and classified as low (<6) or high (≥6) expression.[16]

Statistical Methods

IBM-SPSS 24.0 (IBM-SPSS Inc., Chicago, IL) analyzed and described data through means ± SD/SE, frequencies, and percentages. Normality was tested using the Shapiro–Wilk test. Inferential tests included Chi-square test (for frequency distribution), McNemar, and Friedman tests (for within-group comparisons). Student *t*-test/Mann–Whitney *U* test and one-way ANOVA/Kruskal–Wallis test were used for two-group and multi-group comparisons of continuous data, respectively. RM-ANOVA with Tukey corrections was applied for repeated measures with more than two categories. Weighted Cohen kappa assessed interobserver agreement. Spearman rank correlation was used for association testing. A $p < .05$ was considered statistically significant.

Results

This study was performed on 42 patients with stable vitiligo, whose ages ranged from 18 to 65 years, with 76.2% of patients being females. Demographic and clinical data of patients are summarized in **Supplemental Digital Content 1, Table S1**, http://links.lww.com/DSS/B937. Table 1 demonstrates significant reductions in modified VASI score in all groups after treatment and follow-up ($p < .001$), without significant differences between them. The mean percent reduction in VASI at 3-month follow-up was 28.1 ± 1.1 and 28 ± 1.7 in 100 mJ-FCL + MTX and 5-FU groups, respectively, and 23.94 ± 0.7 and 17.6 ± 0.4 in 50 mJ-FCL + MTX and 5-FU groups, respectively. This improvement was greater than percent VASI reductions recorded at 1 month after treatment. Change in VASI, posttherapy, correlated negatively with age in both MTX ($\rho = -0.361$, $p = .047$) and 5-FU ($\rho = -0.397$, $p = .038$) groups and correlated positively with baseline VASI in 5-FU groups only ($\rho = 0.253$, $p = .049$).

Distribution of treated vitiligo patches across different anatomic sites showed no statistically significant differences among all groups ($p = .202$) (See **Supplemental Digital Content 1, Table S2**, http://links.lww.com/DSS/B937). Lesion site significantly influenced treatment outcomes ($p = .015$), with hands and feet exhibiting the lowest improvement in modified VASI (See **Supplemental Digital Content 1, Table S3**, http://links.lww.com/DSS/B937). Table 2 (Figures 1 and 2) demonstrates degrees of repigmentation assessed by blinded photographic evaluation. The two evaluators provided a substantial agreement ($\kappa = 0.67$; SE = 0.039; 95% CI: 0.60–0.75). A significant difference was found between treatment groups at 1 month after treatment ($p = .007$) with higher excellent improvement grades in 100 mJ-FCL+5-FU group. Instead, at 3-month follow-up, there was no longer a significant difference between groups ($p = .137$). However, significant continued improvement occurred in MTX groups only at follow-up compared with 1-month post-treatment.

Regarding patients' satisfaction toward treatment, there were statistically insignificant differences among studied groups ($p = .747$). The satisfied and very satisfied grades were 60.8% in 100 mJ-FCL + MTX group, 54.2% in both energy-FCL+5-FU groups, and 47.8% in 50 mJ-FCL + MTX group. Monthly follow-up laboratory investigations during study showed that all values remained within normal range except for 2 patients in MTX and 1 patient in 5-FU group who experienced mild elevations in liver enzymes after second treatment, which resolved spontaneously by final follow-up investigations 1 month after treatment. Also, 2 patients in 5-FU group had a slight decrease in hemoglobin level after two sessions, for which they were prescribed supportive treatment without further decrease at final follow-up.

For main adverse effects between MTX and 5-FU treated groups, that is, erythema, pain, and hyperpigmentation, there were statistically insignificant differences. Hyperpigmentation was recorded for 14 (66.7%) patients versus 8 (38.1%) patients in MTX and 5-FU groups, respectively. Only pain was more severe during sessions on higher-energy FCL side in both treatment modalities, whereas degrees of hyperpigmentation and erythema were similar in higher-energy and lower-energy lasers.

Table 3 summarizes treatment effects on serum IL-23 levels. Significant reductions occurred in both MTX and 5-FU groups posttherapy ($p = .001$ and .005, respectively), with greater reductions evident in MTX group ($p = .033$). Similarly, lesional IL-23 expression post-treatment showed significant reductions in all groups (all $p < .001$). Larger percent reductions were shown in groups receiving higher energy FCL with either MTX or 5-FU (Table 3, Figure 3).

Discussion

FCL aids in vitiligo treatment through multiple mechanisms, importantly enhancing topical drug absorption by creating microscopic treatment zones, with efficacy influenced by their depth and density.[17] Topical 5-FU promotes repigmentation by directly stimulating melanocyte proliferation, inhibiting factors that damage pigment cells, and exerting immunomodulatory effects that help stabilize vitiligo.[18,19] Besides reducing TNF-α–producing T cells, MTX may act by increasing IL-10-producing T cells. MTX can also inhibit IFN-α–induced NF-κB activation through

TABLE 1. Comparison of Modified VASI Between Groups

VASI Mean ± SE	50 mJ-FCL+MTX ($n = 23$)	100 mJ-FCL+MTX ($n = 23$)	50 mJ-FCL+5-FU ($n = 24$)	100 mJ-FCL+5-FU ($n = 24$)	p*
Baseline	0.18 ± 0.014	0.29 ± 0.009	0.19 ± 0.01	0.19 ± 0.008	.430
After treatment %Change	0.15 ± 0.012 16.4 ± 1.0	0.25 ± 0.003 18.2 ± 0.4	0.16 ± 0.003 16.3 ± 1.0	0.13 ± 0.006 23.5 ± 1.5	.280 .699
At follow-up %Change	0.13 ± 0.002 23.94 ± 0.7	0.22 ± 0.005 28.1 ± 1.1	0.16 ± 0.008 17.6 ± 0.4	0.12 ± 0.012 28 ± 1.7	.344 .526
p-value†	**<.001**	**<.001**	**<.001**	**<.001**	

Bold p-values are significant when <.05.
* Between groups.
† Within group.

TABLE 2. Post-treatment Photographic Evaluation in Groups

Repigmentation *n* (%)	50 mJ-FCL+MTX (*n* = 23)	100 mJ-FCL+MTX (*n* = 23)	50 mJ-FCL+5-FU (*n* = 24)	100 mJ-FCL+5-FU (*n* = 24)	*p**
After treatment					
No	3 (13)	0 (0)	4 (16.7)	3 (12.5)	**.007**
Minimal	18 (78.3)	19 (82.6)	15 (62.5)	11 (45.8)	
Moderate	2 (8.7)	3 (13)	3 (12.5)	5 (20.8)	
Good	0 (0)	1 (4.3)	1 (4.2)	1 (4.2)	
Excellent	0 (0)	0 (0)	1 (4.2)	4 (16.7)	
At follow-up					
No	1 (4.3)	0 (0)	5 (20.8)	5 (20.8)	.137
Minimal	11 (47.8)	14 (60.9)	10 (41.7)	6 (25)	
Moderate	7 (30.4)	4 (17.4)	5 (20.8)	6 (25)	
Good	3 (13)	1 (4.3)	3 (12.5)	3 (12.5)	
Excellent	1 (4.3)	4 (17.4)	1 (4.2)	4 (16.7)	
p-value†	**0.001**	**0.041**	0.102	0.116	

No (0%); Minimal (1%–25%); Moderate (26%–50%); Good (51%–75%); Excellent (>75%). Bold *p*-values are significant when <.05.
* Between groups.
† Within group.

adenosine release, leading to further regulation of IL-6 production and oxidative stress.[10,20]

Our study found that all treatment groups (MTX or 5-FU combined with lower or higher energy FCL) resulted in significant and continued improvement by modified VASI, without a statistically significant difference between groups after treatment and at follow-up. On blinded photographic evaluation, results favored higher-energy FCL+5-FU after treatment. At follow-up, although all groups were similar, only MTX groups showed significant improvements compared with those at 1-month post-treatment.

So far, one study in literature has compared topical MTX versus topical 5-FU combined with microneedling in vitiligo.[20] In line with our results, both MTX and 5-FU showed comparable efficacies with insignificant differences between them and produced significantly better outcomes at 3-month follow-up compared with microneedling alone. Improvements by VASI were close to our results; it

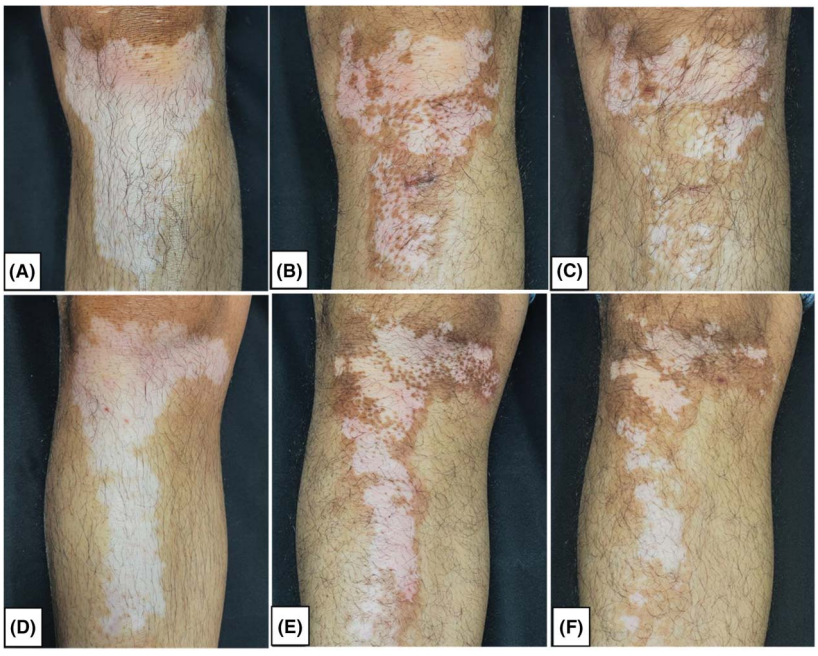

Figure 1. FCL + MTX (legs): Baseline (A and D), 50-mJ energy; Moderate response after treatment (B), Good response at follow-up (C). 100-mJ energy; Moderate response after treatment (E), Excellent response at follow-up (F). N.B. Excellent color match at follow-up.

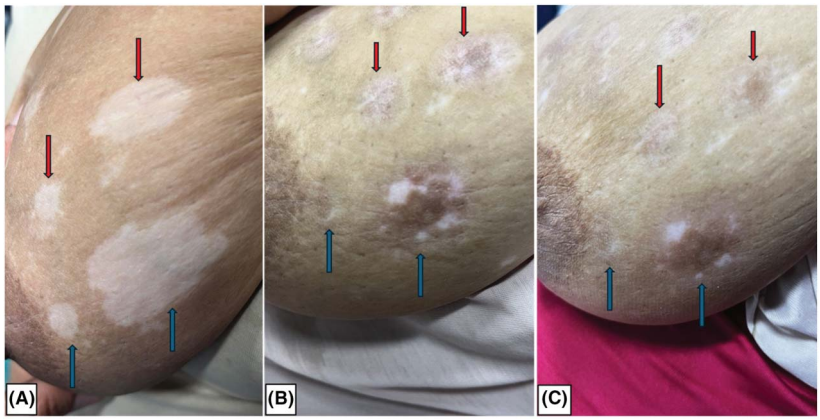

Figure 2. FCL+5-FU (Breast): Baseline (A), 50-mJ energy (red arrow), 100-mJ energy (blue arrow); Excellent response for both after treatment (B), Excellent response for both at follow-up (C). N.B. Good color match at follow-up.

improved by 28.5% in MTX group, 31.03% in 5-FU group, and only 15.8% in microneedling group. Photographic evaluation achieved higher repigmentation grades than our study. Notably, they used a higher MTX concentration than ours. Optimal concentration of topical MTX in vitiligo is still in question. Microneedling enhances transdermal drug delivery by creating microchannels, whereas FCL promotes deeper penetration through controlled ablation of epidermal and dermal layers.[21] Although FCL may facilitate delivery of high–molecular-weight agents,[21] a recent randomized clinical trial in treatment-resistant vitiligo found no significant difference between FCL and microneedling when combined with NB-UVB and topical corticosteroids.[22]

Few studies have assessed efficacy of local MTX in treating vitiligo. Our results were superior to a study that compared microneedling plus topical MTX 1% gel versus microneedling plus topical tacrolimus 0.1% solution. By VASI Score, none of patients showed good/excellent response after treatment in MTX group, and only 10% patients showed good/excellent response at 3-month follow-up.[9] This difference could be attributed to FCL use in our study, which provided better drug delivery to skin and hence, better response.

Elrewiny and colleagues[23] achieved higher improvement rates than our study in patients who received intralesional injections of MTX (50 mg/2 mL) every 2 weeks for up to 6 sessions. They recorded significant clinical repigmentation, with 33.3% patients having good/excellent responses, assessed by photographic evaluation. This can be explained by higher MTX concentration, more frequent sessions, and,

TABLE 3. Treatment Effect on Serum and Lesional IL-23 in Groups

Serum IL-23 (pg/mL), mean ± SE	**FCL + MTX (*n* = 20)**	**FCL+5-FU (*n* = 20)**	***p****
Baseline	3706.36 ± 2117.7	3811.13 ± 2009.2	.056
After treatment	1210.63 ± 635.6	3003.70 ± 1888.8	**.033**
p-value†	**.001**	**.005**	

Lesional IL-23 (IRS), mean ± SE	**50 mJ-FCL + MTX(1), *n* = 12**	**100 mJ-FCL + MTX(2), *n* = 12**	**50 mJ-FCL+5-FU(3), *n* = 11**	**100 mJ-FCL+5-FU(4), *n* = 11**	***p*-value***
Baseline	6.58 ± 0.6	6.58 ± 0.6	4.91 ± 0.6	4.91 ± 0.6	**.040**
After treatment	4.50 ± 0.6	1.67 ± 0.5	2.55 ± 0.6	1.36 ± 0.5	**<.001**
p-value†	**<.001**	**<.001**	**<.001**	**<.001**	
p-value‡	1 versus 2 **<.001**, 1 versus 3 **=.011**, 1 versus 4 **<.001**				
%Change	30.55 ± 3.7	76.85 ± 4.5	51.5 ± 9.2	78.1 ± 6.8	**<.001**
p-value‡	1 versus 2 **<.001**, 1 versus 3 **=.022**, 1 versus 4 **<.001**, 2 versus 3 **=.006**, 3 versus 4 **=.005**				

Bold *p*-values are significant when <.05.
* Between groups.
† Within group.
‡ Interaction between treatment.
IRS, immunoreactivity score.

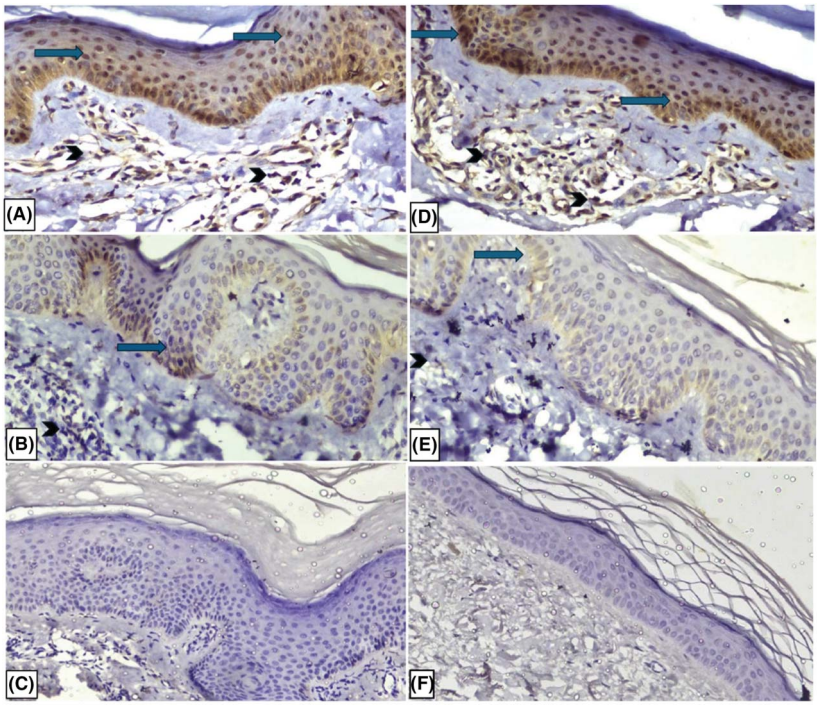

Figure 3. Immunohistochemical IL-23 expression (epidermal keratinocytes-blue arrow and subepithelial inflammatory cells-black arrow). Strong expression at baseline (A and D), FCL + MTX: Mild expression after 50-mJ laser (B), Negative expression after 100-mJ laser (C), FCL+5-FU: Mild expression after 50-mJ laser (E), Negative expression after 100-mJ laser (F).

importantly, a more invasive technique. Our approach using FCL presented a safer cutaneous delivery technique of MTX.

Another study that evaluated topical MTX gel 1% in vitiligo reported that MTX alone achieved good/excellent response in only 6.3% patients by photographic evaluation after treatment. Conversely, MTX combination with NB-UVB or excimer light produced much better outcomes (good/excellent responses in 56.3% and 37.5%, respectively).[10] In our study, monthly laser-assisted topical MTX solution produced close results to daily MTX gel as a monotherapy at 1-month post-treatment, whereas greater results were obtained at 3-month follow-up.

In our study, side effects were generally tolerable, insignificant between groups, and mostly disappeared during treatment or after discontinuation, except for hyperpigmentation. Similarly, the study comparing combined microneedling with 5-FU versus MTX reported that most patients had pain and erythema.[20]

Both MTX and 5-FU significantly reduced serum IL-23 after treatment, with greater reduction observed in MTX. Also, lesional IL-23 immunoreactivity was significantly reduced in all groups posttherapy, with 100-mJ FCL achieving a greater reduction than 50 mJ energy. A study evaluating FCL's role on blood cytokines in vitiligo reported IL-23 to be higher in patients with vitiligo than controls, and similar to our results, its levels significantly decreased after FCL treatment.[6] In addition, another research on 40 patients with vitiligo found serum IL-23 to be significantly lowered after NB-UVB therapy.[24] These findings support existing evidence implicating IL-23/Th17 axis in vitiligo pathogenesis.[25] According to these results, MTX may have a greater effect than 5-FU in reducing IL-23 at a serological level.

This study limitations were the relatively small study sample, few treatment sessions, and short follow-up period, which may not be sufficient to fully report long-term pigment stability and relapse. Also, lack of standardized topical MTX formulation and concentration, and not calculating total VASI for patients are other limitations. However, this research has strengths considering the study design with subgroup analysis and intrapatient comparison. We used an objective, comprehensive multimodal assessment strategy, combining clinical tools with serological and immunohistochemical evaluations.

In conclusion, topical MTX solution applied after FCL offers comparable overall clinical outcomes regarding efficacy and safety to 5-FU for stable, nonsegmental vitiligo. Although 5-FU achieved significant repigmentation earlier, MTX showed a potentially more sustained response after treatment discontinuation. The study suggests a higher energy FCL setting with either treatment to have superior efficacy, both in repigmentation response and immunologic outcomes, specifically lesional IL-23 inhibition. This is the first study to directly assess lesional IL-23 in vitiligo and modulation in tissue expressions along with serum levels after treatment. Serum and lesional IL-23 can be identified as potential markers for therapeutic response in vitiligo.

Recommendations include conducting larger trials with extended follow-up periods to validate long-term efficacy/safety of topical MTX with FCL, comparing combined FCL/MTX to FCL monotherapy, assessing different MTX formulations and laser parameters, exploring combination therapies to enhance results, and further studying IL-23 as

a therapeutic marker in vitiligo. Monitoring laboratory investigations for FCL with topical MTX or 5-FU is judicious, especially when using higher energy setting with a possibility of systemic drug absorption.

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