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ORIGINAL PAPER

Antiangiogenic Effect of Methotrexate and PUVA on Psoriasis

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Abstract Vascular endothelial growth factor (VEGF) is important factor for angiogenesis in psoriasis. Methotrexate and psoralen and ultraviolet light A (PUVA) mainly target the T cell-mediated immunopathology of psoriasis. Our work aimed at estimating VEGF mRNA in psoriatic patients and investigating whether the standard therapeutic modalities (methotrexate and PUVA) exert their antiangiogenic activity through altering VEGF levels. Twenty-four chronic plaque psoriasis patients were enrolled. Patients were divided into two groups (12 patients each); group A received intramuscular methotrexate and group B was treated by PUVA three times/week in a PUVA 1000 cabin for 10 weeks each. Twelve healthy volunteers served as controls. A skin biopsy was taken from lesional skin before and after treatment for RT-PCR detection of VEGF mRNA. Capillary perfusion scanning using LASER Doppler perfusion imaging was performed on the same psoriatic plaque before and after treatment and was also done for the controls. Following both methotrexate and PUVA, a significant reduction in the amount of VEGF mRNA ($P < 0.001$ and $P = 0.002$, respectively) and capillary perfusion ($P = 0.002$) occurred. These reductions were significantly higher in the methotrexate group ($P < 0.001$ and $P = 0.001$, respectively) than in the PUVA group. The percentage of clinical improvement in the examined psoriatic plaque was significantly positively correlated with the percentage of reduction in the amount of VEGF mRNA ($r = 0.850$, $P < 0.001$) and the percentage of reduction in the capillary perfusion ($r = 0.684$, $P < 0.001$). Both modalities may exert an antiangiogenic effect. Methotrexate appears to have possibly a more potent antiangiogenic effect than PUVA.

Keywords Psoriasis · Angiogenesis · VEGF · Capillary perfusion · Methotrexate · PUVA

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Introduction

Neovascularization appears to play an early and important role in the evolution of psoriatic plaques. The elongated tortuous and dilated vessels within the papillary dermis, the increased endothelial surface area and the endothelial cell proliferation are cardinal features of the disease [1]. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been also found to be overexpressed in lesional psoriatic skin and in patients' serum [2, 3].

Methotrexate is a well-known effective therapeutic modality in the treatment of psoriasis. It possesses mainly an antiproliferative action on the keratinocytes, lymphocytes, and other inflammatory cells [4], in addition to an anti-inflammatory effect through reducing the expression of various adhesion molecules and cytokines [5].

However, methotrexate may possess an antiangiogenic activity. Other studies [6, 7] proved that methotrexate inhibits in vitro corneal vascular endothelial cell proliferation and blocks VEGF- and bFGF-induced corneal neovascularization in vivo. Others also demonstrated significant decrease in serum levels of VEGF after 2 and 6 months of

treatment with low dose methotrexate and cyclophosphamide in patients with metastatic breast cancer [8].

Apart from reducing the expression of CD31 (a known endothelial cell marker) in psoriatic lesions [5], the antiangiogenic effect of methotrexate was not studied before in psoriasis and no previous studies have been conducted to evaluate the effect of methotrexate on the expression of VEGF in lesional psoriatic skin.

PUVA therapy is another effective treatment of psoriasis. It acts mainly by suppressing keratinocyte proliferation, inducing their apoptosis, regulating lymphocyte and antigen presenting cell function, and reducing the production of cytokines such as tumor necrosis factor alpha (TNF-α) [9].

The antiangiogenic effect of PUVA therapy has been previously studied. It has been demonstrated that PUVA and UVA can downregulate the expression of VEGF in normal human keratinocytes [10, 11]. Moreover, others demonstrated that UVA causes reduction in the production of VEGF by keratinocytes in cell cultures [12]. Also others showed that PUVA inhibits angiogenesis and induces apoptosis in endothelial cells in vitro [13]. No previous studies have been conducted on the effect of PUVA therapy on lesional VEGF expression in psoriatic patients.

The aim of this study is to evaluate the antiangiogenic effect of both methotrexate and PUVA therapy in psoriasis by measuring capillary perfusion as well as VEGF gene expression in psoriatic lesions before and after treatment with both methotrexate and PUVA.

Subjects and Methods

Patients

The study was carried out on 24 patients with psoriasis vulgaris (18 males and 6 females). Patients were selected from the outpatient clinic of the Dermatology Department, Faculty of Medicine, Cairo University during the period from February 2011 till August 2012. Consent was obtained from the patients before initiation of the study. Also this study was approved by the ethical committee of the faculty of Medicine, Cairo University.

Inclusion and Exclusion Criteria

The inclusion criteria were an extent of psoriasis more than 30 % body surface area justifying treatment with either methotrexate or PUVA therapy and the presence of psoriatic lesions on the dorsum of the hands. Children below 12 years old as well as pregnant and lactating females were excluded. Careful history taking, routine laboratory investigations (including CBC, ESR, blood sugar levels, liver function tests, and kidney function tests), general examination (including peripheral pulsations and measurement of blood pressure), skin examination, ophthalmologic examination, and Doppler examination of the hands were carried out to exclude patients with diabetes, hypertension, peripheral vascular diseases, any form of liver diseases, kidney diseases, severe anemia, bone marrow suppression, cataract, or photosensitive disorders.

Patients were divided non-randomly according to their circumstances into two groups: group 1: methotrexate-treated group, 12 patients (10 males and 2 females). They received weekly intramuscular injections of methotrexate in a dose that varied from 12.5 to 25 mg. Folic acid in an oral dose of 5 mg/day was given to the patients except on the day of the methotrexate injection. Monitoring of the patients was done by doing liver function, kidney function tests, and complete blood picture weekly for the 1st month and then every 2 weeks.

Group 2: PUVA-treated group, 12 patients (8 males and 4 females). They received PUVA sessions, 3 times weekly. The patients received 0.7 mg/kg 8-methoxypsoralen (MOP), 2 h before phototherapy. Each patient was instructed to use sunscreens and protect the eyes during the sessions. Males were instructed to cover the genitalia during the sessions. If thick scales were present, topical keratolytics were given. Monitoring of the patients was done by doing liver function tests and ophthalmologic examination every 4 weeks.

UVA light was delivered by a UV cabin (PUVA 1000, Waldmann, GmbH Germany) equipped with an integrated UV radiometer equipped with F85/100 W fluorescent lamps that emits UV light in the wavelength range of 315–400 nm with a peak emission at 355 nm. The initial UVA dose was dependant on the skin type, and it was 2 J/cm^2 for skin types IV and V and 1 J/cm^2 for skin type III. The dose of UVA was increased by 0.5 J/cm^2 every other session until mild erythema has occurred then the dose was fixed.

Duration of the Study

The study continued until achieving 90 % or more clinical improvement of the lesions or for 10 weeks as an end point.

- Each patient was subjected to the following:
- Psoriasis Area and Severity Index (PASI) score was done for all patients before initiation of therapy (PASI pre-treatment). At the end of the study, PASI score was done (PASI post-treatment) and the percentage of reduction of PASI score was calculated.
- A 4-mm-punch skin biopsy was taken from a particular plaque (lesional skin on the dorsum of the hand) before initiation of therapy and at the end of the study. The

biopsies were stored at −70 °C for quantitative RT-PCR measurement of VEGF mRNA.

- At the end of the study, estimation of the percentage of clinical improvement of the biopsied psoriatic plaque was done.
- Capillary perfusion was measured using the LASER Doppler Perfusion Imaging (LDPI) technique. It was performed before treatment on an area of the same biopsied lesional skin plaque on the dorsum of the hand (perfusion pre-treatment). Two other scans were also done before treatment, one on an area of the perilesional skin and the other on an area of the distal normal skin 2–5 cm away from the psoriatic plaque. At the end of treatment, another perfusion scan was done to the same previously examined area of lesional skin only (perfusion post-treatment).

Controls

Twelve healthy volunteers served as controls (six males and six females). Proper history taking, measurement of peripheral pulsations, blood pressure, laboratory testing for blood sugar levels, and doppler examination of the hands was carried out to exclude subjects with diabetes, hypertension, or peripheral vascular diseases. Consent was obtained from each control and LDPI was done on a circumscribed area of normal skin on the dorsum of the hands.

Methods

RT-PCR for VEGF

Total RNA was extracted from skin biopsies using SV total RNA isolation system (Promega, Madison, WI, USA). The yield of total RNA obtained was determined spectrophotometrically at 260 nm. The extracted RNA was reverse transcribed into cDNA using RT-PCR kit (Stratagene USA). The primers used are specific for amplification of VEGF gene giving 649 bp after PCR amplification. The primers sequences were F- 5′-TCGGGCCTCCGAAACC ATGA-3′ and R- 5′-CCTGGTGAGAGATCTGGTTC-3′.

The cDNA was subjected to PCR amplification using dNTPs, the two primers and *Taq* polymerase enzyme. The PCR cycling condition was 95 °C for 1 min, 64 °C for 1 min, and 72 °C for 1.5 min. This was repeated for 33 cycles. Additional 10 min incubation at 72 °C after completion of the last cycle was performed. VEGF amplification product was detected using agarose gel electrophoresis. The PCR products were then quantitated. Quantitation method depends on purification of the PCR using Promega Wizard PCR preps DNA purification kit (Promega Corporation, Madison, WI, USA). The mixture for quantitation consisted of DNA quantitation buffer, sodium pyrophosphate, NDPK enzyme solution, T4 DNA polymerase, and DNA. All these contents were incubated at 37 °C for 10 min. Then, 100 μL of Enliten L/L reagent was added. Immediately, the reaction was read using a luminometer. The same steps were done on DNAs of known concentrations provided by the kit, and a standard curve was performed by plotting the readings of the luminometer against the concentrations. Then, the readings of the amplified PCR product of VEGF after using the luminometer were read from the standard curve. The results were expressed as (pg/gm tissue) [14].

LASER Doppler Perfusion Imaging (LDPI)

LASER Doppler Perfusion Imaging to measure the capillary perfusion of the psoriatic lesions was done by the PeriScan System (PIM II) (Sweden). The PeriScan PIM II imager comprises a scanning head, Opto-Isolation Unit, and an A/D Data Acquisition Board. The scanning head can be mounted on a mobile cart or with a table mounted arm. Evaluation of results and generation of reports was carried out by the easy-to-use image analysis software (LDPIwin).

Statistical Methods

The statistical package SPSS version 12 was used. The data were measured using mean and standard deviation (SD) for quantitative data and percent for qualitative data. The differences between studied groups were assessed using Chi square tests for qualitative data and analysis of variances (ANOVA) and (Post Hoc), Mann–Whitney test and Wilkson Signed test for quantitative variables. Also, correlation was done to assess the association between two quantitative variables and was expressed as r value. The P value was considered significant if less than 0.05 [15].

Results

The present study included 24 patients, 18 males (75 %) and 6 females (25 %). Their ages ranged from 15 to 60 years with a mean of 38.13 ± 13.8 years. Patients were divided into two groups. The first group, group (1), received methotrexate therapy. They included 12 patients, 10 males (83.3 %) and 2 females (16.7 %) and their ages ranged from 18 to 60 years with a mean of 42 ± 14.96. The second group, group (2), received PUVA therapy. They included 12 patients, 8 males (66.7 %) and 4 females (33.3 %) and their ages ranged from

15 to 56 years with a mean of 12.2 ± 3.53 years. Twelve controls were included in the study, 6 males (50 %) and 6 females (50 %). Their ages ranged from 23 to 54 years with a mean of 9 ± 2.61 years.

Pre-treatment PASI score was statistically homogenous ($P = 0.394$), meanwhile post-treatment PASI score was significantly lower than pre-treatment PASI score in both the methotrexate group ($P = 0.002$) and the PUVA group ($P = 0.002$). However, no statistically significant difference was found when comparing post-treatment PASI scores in both patients' groups ($P = 0.799$) (Table 1). On the other hand, the percentage of reduction in the PASI score and the percentage of clinical improvement of the biopsied psoriatic plaque were significantly higher following methotrexate therapy than following PUVA therapy ($P = 0.021$ and $P < 0.001$, respectively) (Fig. 1).

Both patients' groups were statistically homogenous as regards pre-treatment VEGF gene expression ($P = 0.347$). Post-treatment VEGF gene expression was significantly lower than pre-treatment VEGF gene expression in the methotrexate group ($P < 0.001$) as well as in the PUVA group ($P = 0.002$). Post-treatment VEGF mRNA expression was significantly lower in the methotrexate group than in the PUVA group ($P < 0.001$) (Table 2). The mean percentage of reduction in the amount of VEGF gene expression after treatment with methotrexate (45.3 % ± 3.26) was significantly higher than after PUVA treatment (22.9 % ± 9.47) ($P < 0.001$).

Results of measurement of capillary perfusion are shown in Figs. 2, 3, and 4. Comparison between lesional skin, perilesional skin, and distal skin perfusion in the patients and controls is shown in Fig. 5.

The mean perfusion of psoriatic lesions in the patients as a whole was 1.8 flux (±0.73) while the mean perfusion of the perilesional skin was 1.2 flux (±0.33) and the mean perfusion of the distal normal skin of psoriatic patients was 0.8 flux (±0.18).

In the patients as a whole, the perfusion of lesional skin was found to be ~1.7 times that of the perilesional skin and that difference was highly significant ($P < 0.001$). Moreover, the perfusion of lesional skin was found to be ~2.3 times that of the distal skin and that difference was also highly significant ($P < 0.001$). Yet there was no statistically significant difference between the perfusion of the perilesional skin and that of distal skin in psoriatic patients ($P = 0.105$).

Table 1 Results of the evaluation of the PASI score before and after treatment in both patients' groups

	Methotrexate group	PUVA group	*P* value
PASI (pre-treatment)	12.717 ± 7.37	10.133 ± 4.78	0.394
PASI (post-treatment)	4.892 ± 2.58	4.767 ± 4.13	0.799
P value	0.002	0.002	

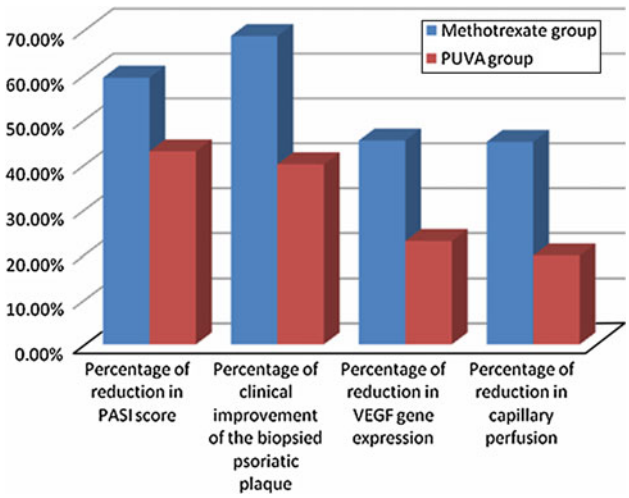

Fig. 1 Comparison between both patients' groups as regards the *percentage of reduction in PASI score*, *percentage of clinical improvement of the biopsied psoriatic plaque*, *percentage of reduction in VEGF gene expression* and *percentage of reduction in capillary perfusion* following treatment

The mean perfusion of the normal skin of controls was 0.67 flux (±0.18). The perfusion of the lesional skin was ~2.7 times that of the controls and the difference was highly significant ($P < 0.001$), and the perfusion of the perilesional skin was ~1.6 times that of the controls and the difference was also highly significant ($P = 0.001$). On the other hand, there was no significant difference in the perfusion when comparing the distal psoriatic skin and the control's skin ($P = 0.268$).

Post-treatment perfusion of the psoriatic lesions was significantly lower than pre-treatment perfusion in the methotrexate group ($P = 0.002$) as well as in the PUVA group ($P = 0.002$). The post-treatment perfusion was significantly lower in the methotrexate group than in the PUVA group ($P < 0.001$). The mean percentage of reduction in the perfusion of psoriatic lesions after treatment with methotrexate was 45 % (±14.28) and in the PUVA group was 19.7 % (±13.25). Such percentage of reduction in the perfusion was significantly higher in the methotrexate group ($P = 0.001$).

The percentage of clinical improvement in the examined psoriatic plaque was significantly positively correlated with the percentage of reduction in the amount of VEGF gene expression ($r = 0.850$, $P < 0.001$) (Fig. 6) and the percentage of reduction in the capillary perfusion ($r = 0.684$, $P < 0.001$) (Fig. 7). Similarly, a significant positive correlation existed between the percentage of reduction in VEGF gene expression and the percentage of reduction in

Table 2 Results of measurement of VEGF gene expression before and after treatment in both patients' groups

	Methotrexate group	PUVA group	*P* value
VEGF mRNA (pg/gm tissue) (pre-treatment)	1824 ± 196.84	1935 ± 251.54	0.347
VEGF mRNA (pg/gm tissue) (post-treatment)	1010 ± 144.81	1498 ± 302.81	<0.001
P value	<0.001	0.002	

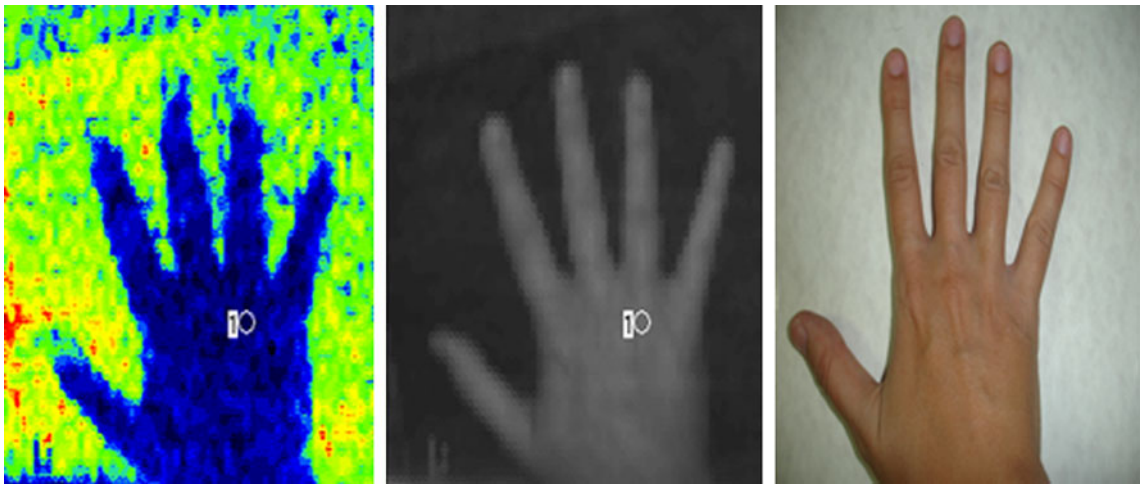

Fig. 2 The perfusion of one of the controls: 0.76

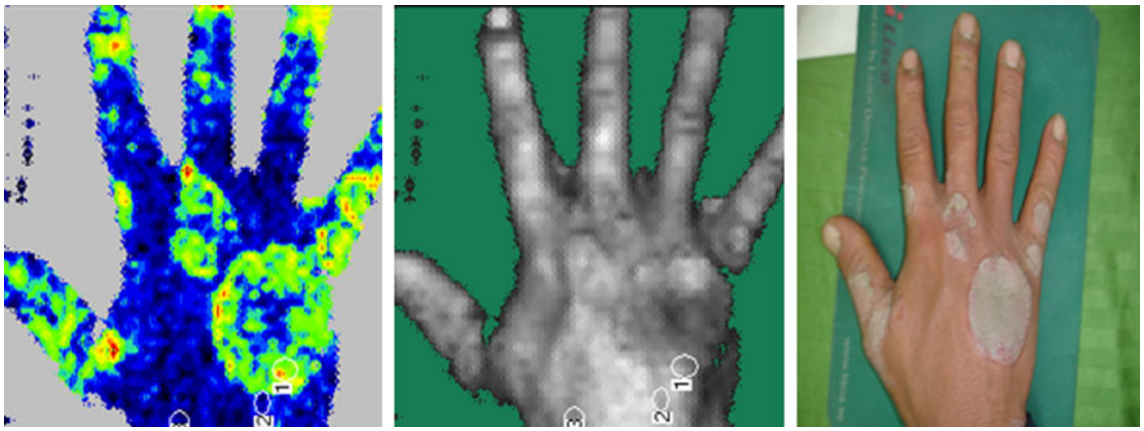

Fig. 3 One of the patients before treatment. Lesional perfusion (1): 2.40, perilesional perfusion (2): 0.92, and distal perfusion (3): 0.72

the capillary perfusion of psoriatic lesions ($r = 0.685$, $P < 0.001$).

Discussion

Our study revealed a 2.7 times more perfusion of lesional psoriatic skin (1.79 flux ± 0.73) than normal skin of controls (0.67 flux ± 0.18) ($P < 0.001$). Perfusion of perilesional skin (1.07 flux ± 0.33) was 1.6 times more than that of the normal skin of controls ($P = 0.001$). These findings are in concordance with a study [16] which used the same LDPI device and demonstrated a 2.8-fold increase in the perfusion of psoriatic lesions compared to normal skin in the same patients. Another study [17], used a dual wave length laser Doppler imaging, and another one [18], by using laser Doppler flowmeter, demonstrated that skin blood flow within the psoriatic plaques was elevated by fourfold than uninvolved skin.

They also demonstrated an area of normal skin with increase perfusion around the psoriatic plaques (2–4 mm) in width [17–19].

Our findings indicate that increased angiogenesis is not limited only to the psoriatic lesion but also normal looking

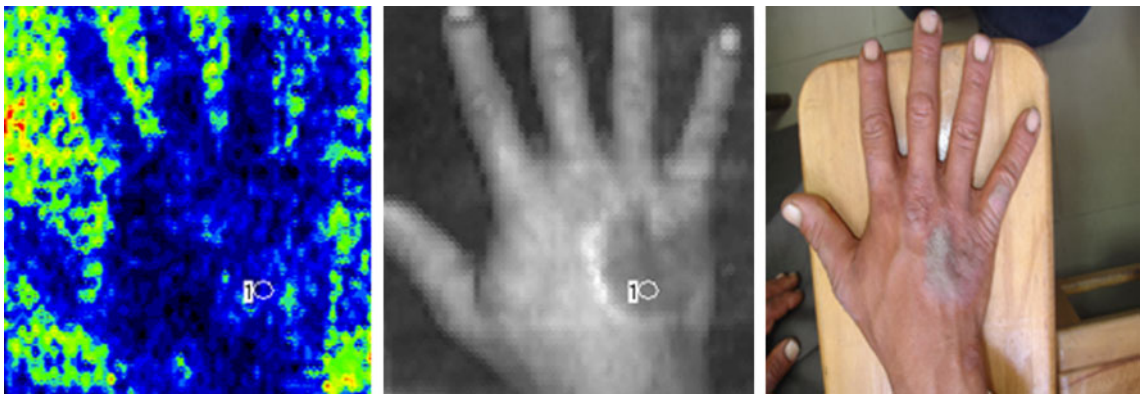

Fig. 4 The same patient after PUVA therapy. Lesional perfusion (1): 2.17

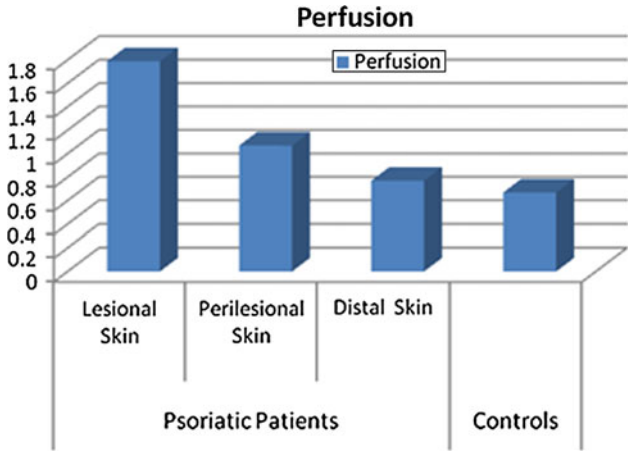

Fig. 5 Comparison of capillary perfusion between lesional skin, perilesional skin, distal psoriatic skin, and normal control skin

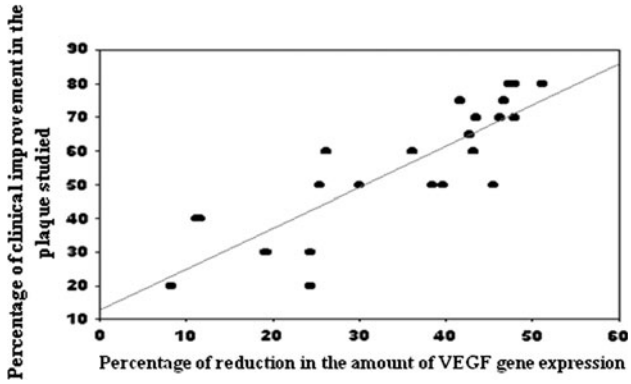

Fig. 6 A significant positive correlation between the percentage of clinical improvement in the examined psoriatic plaque and the percentage of reduction in the amount of VEGF gene expression ($r = 0.850$, $P < 0.001$)

skin, immediately around the psoriatic lesion, exhibits increased angiogenesis. Putting in consideration the well-known peripheral extension of psoriatic plaques, these findings confirms that new vessel formation is an initial step in the evolution of a psoriatic plaque.

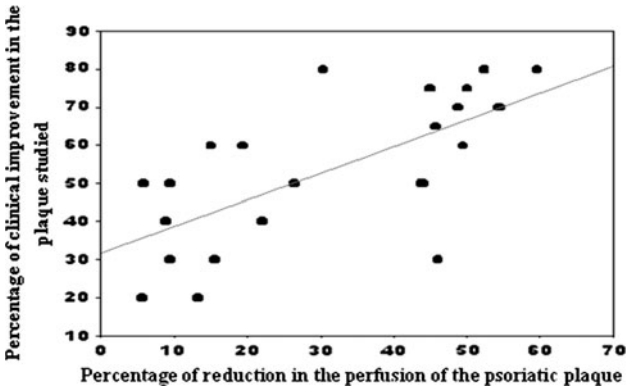

Fig. 7 A significant positive correlation between the percentage of clinical improvement in the examined psoriatic plaque and the percentage of reduction in the capillary perfusion ($r = 0.684$, $P < 0.001$)

In this study, a significant reduction in the PASI score was achieved by both methotrexate ($P = 0.002$) and PUVA ($P = 0.002$). Although the percentage of reduction in PASI score was significantly higher in the methotrexate group ($P = 0.021$), there was no significant difference between both groups as regards post-treatment PASI scores ($P = 0.799$). Also the percentage of clinical improvement of the plaque examined was significantly higher in the methotrexate group than the PUVA group ($P < 0.021$). These results confirm the therapeutic clinical effectiveness of both methotrexate and PUVA in treatment of psoriasis.

A significant reduction in the amount of VEGF expression in lesional psoriatic skin was achieved both in the methotrexate group ($P < 0.001$), and the PUVA group ($P = 0.002$). Similarly, a significant reduction in the perfusion of lesional skin was achieved both in the MTX group ($P = 0.002$) and in the PUVA group ($P = 0.002$). These findings indicate that both methotrexate and PUVA possess antiangiogenic activity. However, the percentage of reduction in VEGF expression was higher in the methotrexate group (45.3 %) than in the PUVA group (22.9 %)

($P < 0.001$), and similarly the percentage of reduction in lesional skin perfusion in the methotrexate group (45 %) was significantly higher than normal controls ($P < 0.001$). In other words, it can be said that methotrexate exhibits a greater antiangiogenic effect than PUVA.

No previous studies have examined the effect of methotrexate on the perfusion of psoriatic plaques. Although several studies have examined the effect of PUVA on perfusion of psoriatic lesions, none of them have used LDPI. In concordance with our findings, a study done [20] using laser Doppler velocimeter, showed that the blood flow in the psoriatic plaques treated with Goeckerman regimen, decreased to levels comparable to those in uninvolved skin and preceded the clinical resolution, whereas the perfusion of the PUVA-treated lesions were consistently higher even despite clinical healing. In another study [21], the cutaneous blood flow measured using laser Doppler flowmeter reduced with PUVA therapy and this reduction was closely related to the clinical improvement, however, the values of the cutaneous blood flow were higher than that of the clinically normal skin even after clinical remission of the lesions. This was proved also by [22] ultrastructural studies in which the skin vessels reduced in length and coiling but the capillary loops remained dilated for months after successful PUVA therapy.

In all patients, the percentage of reduction in the amount of VEGF expression was significantly positively correlated with both the percentage of reduction in the perfusion of the lesions ($r = 0.685$, $P < 0.001$) and the percentage of clinical improvement of the examined plaque ($r = 0.850$, $P < 0.001$). Similarly the percentage of reduction in the perfusion of the lesions was significantly positively correlated with the percentage of clinical improvement in the plaque examined ($r = +0.684$, $P < 0.001$). This further highlights that suppression of angiogenesis is crucial in achieving clinical improvement of psoriatic lesions.

The mechanism by which methotrexate reduces VEGF expression (and consequently perfusion of psoriatic lesions) could be due to either a direct effect of methotrexate on VEGF production from keratinocytes or it could be attributed to the well-known antiproliferative effect of methotrexate on keratinocytes [4], which are the main source of VEGF in psoriasis [23].

It has also been shown that MTX inhibits cytokines as IL-1 and TNF-α which are potent angiogenic factors [24] and that the reduction in the level of these cytokines is not only due to the decrease in the number of inflammatory cells, but also through the conversion of MTX to MTX-polyglutamates, which promotes adenosine release. Adenosine also inhibits the transcription of IL-6 and IL-8 in monocytes and macrophages [25].

The effect of MTX on angiogenesis is controversial and inadequately investigated. In an experiment dealing with in vivo angiogenesis [6] showed no histological evidence of inflammatory and mononuclear cell infiltration around the induced blood vessels (in animal models) treated with MTX and showing marked reduction in the number of these blood vessels, this would indicate that the antiangiogenic effect is due to a direct action of MTX on the vessels and not to alteration of the capability of inflammatory cells for induction of neovascularization. Also MTX did not inhibit DNA synthesis in human ECs lines in vitro, suggesting that its antiproliferative action is not to be implicated in the inhibition of angiogenesis [26].

The mechanism of inhibition of angiogenesis by MTX may be related to modulation of adhesion molecules [27]. In a study conducted [28] in (1995), they showed that MTX might inhibit angiogenesis through the modulation of the neo-angiogenesis mediated by integrin α (5) β (3) and α (v) β (5). MTX also suppresses the expression of ICAM-1 and E-selectin by dermal endothelial cells [29].

PUVA downregulates lymphocyte and antigen presenting cell function. Also it influences adhesion molecules expression and diminishes Langerhans cells number within the epidermis [9]. The inhibitory effect of UVR on DNA synthesis and the suppression of immune system in the cutaneous tissues may contribute considerably to the improvement of epidermal hyperplasia and infiltration of lymphocytes in the dermis [30].

Recently, there are increasing evidences indicating that the inhibition of angiogenesis can be a possible mechanism of PUVA in the treatment of psoriasis, as the initial improvement during PUVA treatment was in the microvessels of the psoriatic lesions [31]. PUVA may reduce VEGF expression through a primary effect on VEGF production by keratinocytes or secondary to its known inhibitory effect on the proliferation of keratinocytes [11].

In conclusion, both methotrexate and PUVA cause reduction in the amount of VEGF expression and the degree of perfusion of psoriatic lesions indicating that both therapeutic modalities may be effective in the treatment of psoriasis via an antiangiogenesis mechanism. And since methotrexate showed more significant reduction in these two parameters we can conclude that methotrexate is a more potent antiangiogenic therapeutic modality than PUVA in the treatment of psoriasis.

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