Original Article

Genotoxicity assessment using micronuclei assay in vitiligo patients treated with narrow-band UVB

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Abstract

Background: The treatment of vitiligo is still a challenge. Among various therapeutic modalities, phototherapy with ultraviolet B narrow-band(UVB-NB) is presently considered a treatment of choice for this disease.The narrow-band UVB(TL01) lamp (311nm emissions) was developed as an alternative to a broad-band UVB source and to photochemotherapy, both of which have significant side-effects and carry a risk of carcinogenesis. The micronucleus(MN) assay in peripheral blood lymphocytes was used as a sensitive method for measuring genetic damage in human populations exposed to genotoxic agents .

Aim of Work:: to measure genetic damage by evaluating micronucleus frequencies in mitogen-stimulated lymphocytes of patients with vitiligo treated by narrow-band UVB.

Subjects and Methods: The current study was conducted on 20 patients with vitiligo (mean age: 20.7±12.1 years) to evaluate frequency of micronuclei in 72h cultivated/mitogen-stimulated lymphocytes at pretreatment, and after 20, 40, 60 sessions of narrow-band UVB treatment. MN values were scored in binucleated cells obtained from cultivated/mitogen-stimulated lymphocytes of patients.

Results: While the beginning MN frequency

Key words: Micronuclei, vitiligo, narrow-band UVB

± SD (%) was 1.05 ± 0.63, it increased to 1.46 ± 0.67, 1.47 ± 0.54, 1.4 ± 0.33 corresponding, respectively, to 20, 40, 60 sessions. These sessions reciprocally correspond to 1.130 ± 0.8, 2.42 ± 1.0, 3.97±1.0 J/cm² doses of narrow-band UVB. Difference of MN frequency was statistically significant ($p<0.05$). The relationship between the MN frequency and narrow-band UVB doses was not statistically significant ($P > 0.05$), and there was no dose-dependent effect on increase of MN values.

Conclusion: Our results showed that narrow-band UVB treatment causes a detectable chromosome damaging effects, which are known to be the initial genetic changes in the tumor genesis of UV-induced skin cancer.

Introduction

Vitiligo is an acquired cutaneous disorder of pigmentation with a 1% to 2% incidence worldwide, without sex or skin color predilection[1]. Clinically, it is characterized by the development of depigmented macules or patches with clear aces, that may show a localized, segmental or generalized distribution[2,3]. Several hypotheses have been proposed for the aetiology and pathogenesis of vitiligo, but the cause remains unclear[4].

The treatment of vitiligo is still a challenge. Among various therapeutic modalities, phototherapy with ultraviolet B narrow-band (UVB-NB) is presently considered a treatment of choice for this disease[1]. It is as an alternative to a broad-band UVB source and to photochemotherapy, both of which have significant side-effects and carry a risk of carcinogenesis[5].

Measurement of micronucleus frequency in peripheral blood lymphocytes is extensively used in molecular epidemiology and cytogenetics to evaluate the presence and the extent of chromosomal damage in human populations exposed to genotoxic physical/chemical agents[6]. The high reliability and low cost of the MN technique, has contributed to the worldwide success and adoption of this biomarker for in vitro and in vivo studies of genome damage[7].

In the present study , we tried to evaluate MN frequencies in mitogen-stimulated lymphocytes of vitiligo patients treated with narrow-band UVB, and correlate it with clinical response.

Subjects and Methods

We recruited 20 vitiligo patients to the study from the outpatient clinic of the Dermatology &Venereology Department, Zagazig University during the period from june 2005 to june 2006.

Criteria for exclusion included: pregnancy; lactation; skin typeI; phototherapy within past six months; history of smoking; cutaneous photosensitivity; skin cancer; patients with a history of photomediated disorders; concomitant radiotherapy; chemotherapy or immunosuppressive therapy and claustrophobia.

All of the patients were treated with UVB-NB phototherapy thrice a week, never on two consecutive days, and always shielding genitals and eyes (unless affected by vitiligo). If the lesions improved, treatment sessions were decreased to twice a week. Informed written consents were obtained from the patients. All patients received comprehensive information regarding nature of therapy.

Eight mercury low pressure lamps (Philips TL-01) with a spectrum of 305-315nm, Maximum wavelength of 311nm were installed in a Cosmedico Cabinet (Medizintechnik, Schwenningen, Germany)was used.

Initial doses and increments were given

according to skin phototype using ready calibrated tables supplied by the manufacturer (Cosmedico Medizintechnik, Schwenningen, Germany). If there was no erythema after starting dose, a 20% increment was used for the first four doses, followed by 0% increment for two sessions, then 10% increment. If the patient reported mild erythema or pruritus, the irradiation dose was held constant for the subsequent treatment or until resolution of the symptoms. If the erythema was marked, the irradiation dose was decreased by 15% or transitorily interrupted.

Patients were advised to protect their skin against excessive exposure to natural sunlight especially between 11 a.m. and 3 p.m. during sunny days on both treatment as well as no-treatment days.

Photographs of the body sites affected by vitiligo were taken for each patient before and after the end of 60 sessions. Follow-up was done for 6 months after completion of treatment to asses the stability of lesional repigmentation. Patients were monitored for repigmentation. The clinical response to therapy was visually scored as the percentage of repigmentation of the depigmented lesions according to Njoo[8], with a numeric value according to the obtained repigmentation grade: 0(no repigmentation), 1 (repigmentation lower than 25%), 2 (repigmentation between 25% and 75%), 3 (repigmentation higher than 75%), 4 (complete repigmentation).

Lymphocyte cultures and MN analysis:

Blood samples were obtained from each subject by venipuncture in heparinized vacutainers, coded and sent within 24 h to the laboratory where they were processed. Lymphocyte cultures were set up by adding 0.5 ml whole blood to 4.5 ml RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1% antibiotics (penicillin and streptomycin) and L-glutamine. Lymphocytes were stimulated by 1% phytohaemagglutinin (all obtained from Gibco laboratories) and incubated for 72 h at 37°C. Two cultures per subject were established. A final concentration of 6 μg/ml cytochalasin B (Sigma, St Louis, MO) was added to the cultures 44 h later to arrest cytokinesis. At 72 h of incubation, the cultures were harvested by centrifugation at 800 r.p.m. for 8 min and treated with an hypotonic solution (2–3 min in 0.075 M KCl at 4°C). Cells were centrifuged thereafter and a 3:1 (v/v) methanol: acetic acid solution was gently added. This fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative solution and dropped onto clean slides. Finally the slides were stained with 10% Giemsa in phosphate buffer (pH 6.8) for 10 min and scored.

To determine the frequency of binucleated cells with micronuclei and the total number of MN in lymphocytes, a total of 1000 binucleated cells with well preserved cytoplasm (500 per replicate) were scored per subject on coded slides. This is the number of cells usually scored in most laboratories[9]. For the scoring of micronuclei the following criteria were adapted from Fenech et al[10].

- The diameter of the MN should be less than one-third of the main nucleus.
- MN should be separated from or marginally overlap with the main nucleus/nuclei as long as there is clear identification of the nuclear boundary.
- MN should have similar staining as the main nucleus/nuclei.

Statistical analysis:

Data were coded, checked, entered and analysed using SPSS II. Data were expressed as mean± SD for quantitative variable, number and percentage for qualitative one. Student t test, paired t test, chi-squared (x^2) and Correlation Coefficient were used. $P < 0.05$ was considered significant.

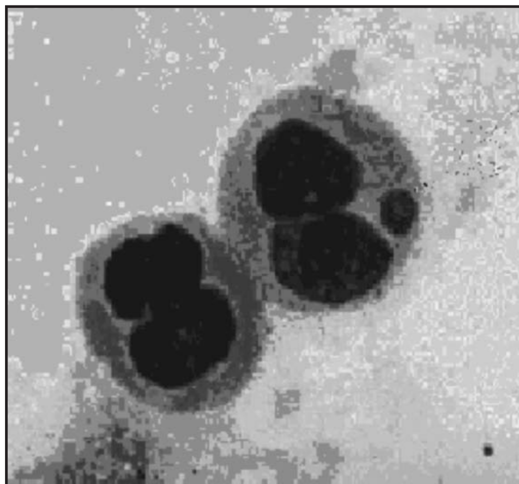

FIG .1. The micronucleus assay showing the binucleated cells with main nuclei that touch and even overlap.

Results

A total of 20 vitiligo patients , fourteen females and six males were recruited to the study. Their ages were ranging between 7 and 52 years with a mean of 20.7±12.1 years. Five of them had skin phototype II, 7 had skin phototype III, and 8 had skin phototype IV (according to Fitzpatrick classification I-VI[11]. After 60 sessions of treatment, all the lesions achieved a variable degree of repigmentations. Complete cure of the lesions was noted in 4/20(20%) of patients. Five patients (25%) showed >75% of repigmentation, 5 patients showed 25-75% of repigmentation, 5 patients showed <25% of repigmentation and only one patient showed no pigmentation (table 1). No new lesions appeared in any patient during the follow up period. The number of MN in at least 1000 binucleated cells (Fig.1) was scored, and the frequency of percentage MN was calculated for each patient at pretreatment, the 20th, 40th and 60th sessions. While the beginning MN frequency ± SD(%) was 1.05± 0.63 (n=20), it increased to 1.46±0.67(n=20), 1.47±0.54 (n=15), 1.4±0.33 (n=11) at the 20th, 40th, 60th sessions respectively. This increase in the MN frequency was statistically significant (P<0.05) between initial MN frequency and 20th, 40th,and 60th sessions. However, no significant difference was found between 20th,and 40th sessions, 20th and 60th sessions, and 40th and 60th sessions (P > 0.05) (table2). No significant correlation was found between duration of the disease and MN frequency at pretreatment (r =0.14, P >0.05), while, the relationship between age of patients and MN frequency before treatment was statistically significant (r =0.42, P<0.05) (Table3). The results of narrow-band UVB doses in different sessions were presented in (Table 4). The 20th session was 1.130±0.8 J/cm^2, the 40th session was 2.42±1.0 J/cm^2,and the 60th session was 3.97±1.0 J/cm^2 of narrow-band UVB doses. Negative correlation between repigmentation and MN frequencies at 20, 40, 60 sessions was not significant (r =-0.02, r =-0.06, r =-0.08, P >0.05). Correlation between the MN frequency and dose at 20, 40, 60 sessions was not statistically significant (r =0.01 ,r =0.33 ,r =0.29, P >0.05)(table5).

Discussion

The use of narrow-band UVB phototherapy for vitiligo was first reported by Westerhot and Nienweboer-Krobotova in[12]. Now, it is considered the gold standard for vitiligo[13]. Narrow-band UVB results in less irritation and erythema and is more effective than broad-band UVB.

The mechanism of action of NB-UVB for treatment of many inflammatory dermatoses is thought to be through the induction of apoptosis and great depletion of T cells. DNA damage is one of the major molecular triggers for UVB-induced apoptosis[14]. Caspases, which are apoptosis associated Serine proteases within the cell, are activated and cause a cascade of events that trigger nuclear condensation, DNA fragmentation, and disintegration of the cell[15]. Induction of apoptosis by UVB, however, is not specific for keratinocytes but affects other cells as well including lymphocytes and macrophages[16]. NB-UVB induces apoptosis in T lymphocytes more efficiently than BB-UVB[14]. Therefore,the T-cell apoptosis-inducing capacity of UVB

light source can be paralleled by its clinical efficacy[17].

Little information is available on the carcinogenic risk of NB-UVB phototherapy in humans. UVB (290-320nm) cause different kinds of cellular DNA damage and mutations within skin cells. This damage is generated directly through absorption of energy. Mutations caused by UV-induced DNA damage are known to be the initial genetic changes in the tumor genesis of UV- induced skin cancer[18].

Murine studies, when collectively analysed, indicate that the cancer risk was higher with treatment NB-UVB than with BB-UVB, but in patients the minimum erythema dose equivalent dose of NB-UVB given to get the same result is lower, and therefore BB-UVB and NB-UVB are considered to have the same carcinogenic risk[19]. Also,NB-UVB associated skin cancer risk may be less than that with PUVA[20].

Several strategies have been suggested to reduce carcinogenicity during UVB phototherapy[21,8][20-25], including:

- Skin saving procedure; parts of the body where no lesions are present should be shielded during treatment; genitals should be also shielded because these areas, as a rule do not respond to phototherapy and genital tumors have been observed after PUVA therapy.
- Prevention of unnecessary exposure to natural sunlight on both treatment and non-treatment days, and use of UV-blocking agents on sun exposed areas.
- The use of combined treatment with other modalities to reduce the cumulative dose.
- Chemoprevention by using nontoxic diet with antitumor properties.
- Less frequent doses
- Light dose adjustment; near erythemogenic doses of NB-UVB, clear psoriasis faster than lower doses of NB-UVB, but the later regimen is equally effective with only slightly more treatments.

The MN assay is simple and allows a measure for both whole chromosome loss and chromosome breaks. This assay can be used for detecting chromosome damage induced in vivo by both Clastogens and physical/chemical agents[9,26]. In the present study, we demonstrated that narrow-band UVB is an inducer of MN in human lymphocytes after therapy. Narrow-band UVB treatment led to increase in MN frequency in lymphocytes of vitiligo patients after that of 20, 40, 60 sessions ($P < 0.05$). No significant increase in MN frequencies between 20, 40/ 20, 60/ or 40, 60. Thus, these results showed that lymphocytes are damaged from radiation, regardless of the increase in narrow-band UVB doses. This might be due to the decrease of the treatment frequencies, after the observation of repigmentation. During this time interval, there could have been enough time for repairing the damaged DNA.

Our data indicated an increase in MN frequencies in lymphocytes dependant on DNA damage after narrow-band UVB therapy. It could be postulated that narrow-band UVB has more therapeutic effect for vitiligo because narrow-band UVB light is more effective than broad-band UVB in inducing T-cell apoptosis[17]. Therefore, increased MN frequencies in stimulated lymphocytes of vitiligo patients after narrow-band UVB therapy may be presented as a novel approach to autoimmune hypothesis in vitiligo pathogenesis[4].

MN frequencies was significantly increased with age ($r = 0.42$, $P < 0.05$), as previously reported[27].

The results of our study supported the previous report that narrow-band UVB is associated with chromosome damage and this damage can trigger apoptosis[27], thus narrow-band UVB treatment might have carcinogenic effect. Therefore, physicians should be careful with using this treatment, over long term. Further controlled studies will be continued to confirm these findings for safety of the patients.

Table (1): Demographic and clinical data of the studied group.

	N = 20
Age (years)	
Median (Range)	18 (7-52)
$\bar{X}$±SD	20.7±12.1
Gender	
Male/Female	6/14 (30.0%/70.0%)
Duration of disease	
Median (Range)	1.5 (2 month – 12 years)
$\bar{X}$±SD	3.1± 3.69
Skin phototype	
II	5 25.0%
III	7 35.0%
IV	8 40.0%
Repigmentation grade	
0 (no pigmentation)	1 5.0%
1 (<25%)	5 25.0%
2 (25-75%)	5 25.0%
3 (>75%)	5 25.0%
4 (complete cure)	4 20.0%

Table (2): MN frequencies of the patients at 20, 40, 60 sessions of narrow-band UVB treatment in comparison with their pretreatment values.

Treatment session	n	$\bar{X}$±SD	Range
Before treatment		1.05±0.63*	0.37-2.78
After 20 sessions	20	1.46±0.67	0.48-2.98
Before treatment		0.99±0.61+	0.35-2.71
After 40 sessions	15	1.47±0.54	0.49-2.99
Before treatment		0.92±0.50*	0.39-2.59
After 60 sessions	11	1.4±0.33	0.75-1.88

*P<0.05 when compare with pretreatment
+P<0.001 when compare with pretreatment
No significant differences between 20th, 40th & 20th, 60th & and 40th, 60th sessions

Table (3): Correlation between MN frequencies and age of the patients, duration of the disease before treatment.

	r	P	Significant
Age of the patients	0.42	<0.05*	.Sig
Duration of the disease	0.14	>0.05	NS

Table (4): Narrow-band UVB doses at 20, 40, 60 sessions of treatment according to skin phototypes.

Treatment session	Skin types (n)			Mean dose±SD (J/cm²)
	II (5)	III (7)	IV (8)	
20	0.995±0.49	1.14±0.78	1.26±1.1	1.130±0.8
40	1.755±0.85	2.37±1.0	3.1±1.3	2.42±1.0
60	2.02±0.79	3.63±0.9	5.44±1.16	3.97±1.0

Table (5): Correlation of MN frequencies and repigmention, cummulative dose of narrow-band UVB at 20, 40, 60 sessions of treatment.

	20		40		60	
	r	P	r	P	r	P
Repigmentation	-0.02	>0.05	-0.06	>0.05	-0.08	>0.05
Cumulative dose	0.01	>0.05	0.33	>0.05	0.29	>0.05

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