EVALUATION OF THE CONDENSED CHROMATIN INDEX IN MITOGEN-STIMULATED LYMPHOCYTES PUVA TREATED PATIENTS*
PUVA Tedavisi Gören Hastaların Mitojenle Uyarılmış Lenfositlerindeki Yoğunlaşmış Kromatin İndeksinin Değerlendirilmesi

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Summary : *Photochemotherapy with psoralens and UV-A (PUVA) is a treatment used widely in some skin disease like as vitiligo and psoriasis. PUVA treatment induces also chromatin condensation and apoptosis in several cell types. The aim of this study is to assay if the condensed chromatin index (CCi) in stimulated lymphocytes of PUVA treated patients may be used as a test method for the detection of DNA damage like the micronucleus (MN) and the sister chromatid exchange (SCE) methods. A special image analysis procedure has been designed to evaluate CCi in the nucleus. The work was initiated with the Mitogen- stimulated lymphocytes of 20 PUVA treated patients. The mean CCi (CCmi) have been evaluated at zero time (n=20) and after 20 (n=16), 40 (n=12), 60 (n=10) sessions of PUVA treatment in 100 mitogen- stimulated interphase nuclei for each patient. These sessions correspond reciprocally to 57.67 ± 23.74, 181.43 ± 46.42 and 299.56 ± 61.03 joules/cm^2 of UVA and 0.6x20, 0.6x40, 0.6x60 mg/kg of methoxypsoralen (8-MOP). There was no difference between CCmi obtained before treatment (zero time) and after 20, 40 sessions of PUVA treatment (p> 0.05). However, statistically significant difference was found between untreated and 60 sessions treated individuals (p<0.05). CCi sensitivity begins after the moderate term of PUVA treatment corresponding to nearly 300 Joules/cm^2 of UVA and 0.6x60 mg/kg of 8-MOP.*

Key words: *PUVA, Condensed chromatin, image analysis*

Özet : *UV-A ve psoralenlerle birlikte kullanılan fotokemoterapi (PUVA) vitiligo ve psoriasis gibi bazı deri hastalıklarında yaygın olarak kullanılan bir tedavidir. PUVA tedavisi aynı zamanda çeşitli hücre tiplerinde apoptosis ve kromatin yoğunlaşmasına neden olmaktadır. Bu çalışmanın amacı PUVA ile tedavi edilen hastaların mitojenle uyarılmış lenfositlerinde yoğunlaşmış kromatin indeksinin (YKi) , kardeş kromatid değişim (KKD) ve mikronukleus (MN) metodları gibi DNA hasarlarının belirlenmesinde bir test olarak kullanılıp kullanılmayacağını tespit etmektir.Çekirdekteki kromatin yoğunluğunu ölçmek için özel bir bilgisayar programı yapıldı. Çalışmaya PUVA tedavisi alan 20 hastanın mitojenle uyarılmış lenfositleri ile başlandı. Her hastanın sıfırıncı saatte (n=20) ve tedavinin 20'ci (n=16), 40'ıncı (n=12) ve 60'ıncı (n=10) seans sonrası, mitojenle uyarılmış 100 interfaz çekirdeği YKi ortalaması değerlendirildi. Bu seanslar sırasıyla 57.81 ± 23.85, 181.43 ± 46.42, 299.56 ± 61.03 jul/cm^2 UVA and 0.6x20, 0.6x40, 0.6x60 mg/kg methoxypsoralen (8-MOP)'e karşılık gelmektedir. Tedavi öncesi (sıfırıncı saat) ile 20. ve 40. seans sonrası elde edilen YK değerleri arasında bir fark bulunamamıştır (p> 0.05). Bununla birlikte , tedavi öncesi ile 60. seans sonrası arasında istatistiksel olarak önemli bir fark bulunmuştur (p<0.05). YKi 'de duyarlılık, yaklaşık 300 jul/cm^2 UVA ve 0.6x20, 0.6x40, 0.6x60 mg/kg 8-MOP'a karşılık gelen PUVA tedavisinin ortalarından sonra başlamaktadır.*

Anahtar kelimeler: *PUVA, Yoğunlaşmış kromatin, bilgisayarla analiz*

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The term PUVA (Psoralens plus Ultraviolet A) describes a form of treatment, embracing psoralens, a group of furocoumarin compounds and exposure of the patients to a source of UV light of 320-400 nm, used for several years as an effective photochemotherapy of some human skin disease such as psoriasis and vitiligo (1).

PUVA treatment induces apoptotic cell death in lymphocytes. Apoptotic degeneration of the epidermal cells after PUVA treatment has been reported firstly by Hashimoto and Kumakiri (2). Subsequent studies established that PUVA treatment induces apoptosis of infiltrating inflammatory cells that are responsible for the formation of psoriatic lesions (3 - 5). Apoptosis is characterized by morphological changes such as cell shrinkage, chromatin condensation and DNA fragmentation (6). PUVA therapy leads also to mutagenic and a possible carcinogenic effect (7). Mutagenic agents cause also some chromatin condensation in interphase nuclei (8,9). We have designed a computer program to measure numerically condensed chromatin surface (CCs)/ total nuclear surface (TNs) proportion (10) defined here as condensed chromatin index (CCi). To our knowledge, no data have yet been reported on the CCi evaluation in interphase nuclei of PUVA treated patients. In the present study, we report CCi evaluation in mitogen-stimulated human lymphocytes treated with PUVA .

MATERIALS AND METHPODS

Patients : 20 patients between aged 13-52 resistant to conventional treatment were selected for the PUVA treatment from Dermatology clinic of Medical Faculty of Erciyes University between 1997-1999. Six of them were diagnosed as psoriasis, 8 as vitiligo and 6 as alopesi areata. Eight patients after 20 sessions and 5 after 40 sessions abandoned the treatment. Because they have not returned for the control, their remission statuses have not been evaluated. Patients were excluded under the following conditions: a history of previous side effect or phototoxic reactions related to photo (chemo) therapy, a history of photosensitivity or photomediated disorders, skin type I (according to Fitzpatrick classification I-VI) (11), concomitant radiotherapy, chemotherapy, or immunosuppressive therapy, and claustrophobia. A questionnaire collecting information on past medical history, alcohol, drug and smoking habits was completed for each patient on the days of the examination.

Ethical Consideration : The local ethics committee approved the study protocol. The study was conducted in accordance with the declaration of Helsinki and local laws, depending on which afforded greater protection to the patients. All study procedures, including details of the possible benefits and side effects had been explained, and written informed consent obtained before enrolment.

PUVA Therapy : 8-MOP 0.6-0.7 mg/kg was given orally at the beginning of each session. Two h later, UVA irradiation of the body surface was followed by a cabin Waldman UV 8001 K. At the beginning, patients received 0.5 joules (J) UVA/ cm^2. This dose was increased regularly 0.5 J after each of 3 sessions of treatment. The treatment was repeated 3-4 days a week until the maintenance therapy was started. Maintenance therapy was administered once or twice a week (≈ 7J or 14 J / cm^2) for an average of 4 months or more. If the erythema had appeared during the treatment, the dose would have been decreased or not increased depending on the skin type and the clinical state of the patient.

Lymphocyte Culture : Peripheral blood was obtained aseptically from patients at zero time and after different sessions of treatment. 0.3 ml whole blood was added to 5 ml Ham's F10 medium (Biological Industry, briefly Biol. Ind.) enriched with 30 % fetal calf serum (Biol. Ind.) 2 % v/v phytohemagglutinin (PHA) (Biol. Ind.), 1% v/v

penicillin-streptomycin (Biol. Ind.) and 1% heparin in sterile plastic culture tubes.

Cells were harvested at the end of 72h. After treatment with hypotonic solution (0.075 M KCl for 20 min) and fixation in three changes of methanol-acetic acid (3:1), spreading on the slide, drying and GIEMSA staining were followed.

Mean Condensed Chromatin Index (CCmi) of the Patients : The calculation of the condensed chromatin surface (CCs)/total nuclear surface (TNs) of giemsa stained cells by image analysis were given previously (10).

Obtaining the images: Before analysing of cells images obtained from a light microscope via microscope camera and a video capture card, they were saved to the disk as a bitmap file. In the first step a simple tone standardisation algorithm were used. *Determining and analysing condensed chromatin area:* Condensed chromatin areas were determined manually by user. For this, user selects firstly a threshold value between "0" to 255. Program selects neighbouring pixels on the image texture according to the threshold value. User can deselect a selected area or select an unselected area. After marked selected area as condensed chromatin area, user selects nucleus in the same way. Number of pixels in the marked area as condensed chromatin area determined as condensed chromatin surface value. Number of pixel in the selected nucleus area is determined as nucleus surface value (10). CCs/TNs was taken as CCi of a given nucleus. The mean of CCi of 100 fully activated nuclei [nuclear diameter of 17 µm or more in flattened state on the slide (12) was taken as mean condensed chromatin index (CCmi) for each patient.

Statistical Analysis : Statistical comparisons on mean values of CCmi frequency before and after treatment were done using paired-sample t test and Wilcoxon tests. Correlations analysis was used to assess the relationship between CCmi value and UVA (J /cm^2) doses. Significant level was set at $p<0.05$.

RESULTS

The individual data of 20 patients are shown in Table I. Mean age ± SD was 25.35±10.00 in the range of 13-52. The table II shows the means of CCmi of patients at zero time (n=20) and after 20 (n=16), 40 (n=12), and 60 (n=10) sessions of PUVA treatment. Hundred fully activated lymphocytes nuclei (nuclear diameter of 17 µm or more in flattened state on the slide) have been evaluated for each case. These sessions correspond reciprocally to 57.67 ± 23.74, 181.43 ± 46.42, 299.56 ± 61.03 joules/cm^2 of UVA and 0.6x20, 0.6x40, 0.6x60 mg/kg of 8-MOP. There was no difference between CCmi obtained before treatment (zero time) and after 20, 40 sessions of PUVA treatment (p=0.70, p=0.24 respectively by paired-sample t test and p=0.96, p=0.27 by Wilcoxon test, Table II), but a statistically significant difference was found between untreated and 60 sessions of PUVA treatment (p= 0.032 by paired-sample t test and p=0.028 by Wilcoxon test, Table II). Large interindividual variation concerning CCmi before and after each session of treatment is apparent.

The correlation between the CCmi and dose is not found statistically significant (p=0.15), and there is no dose-dependent effect on increase of CCmi values.

Table I. Characteristics and CCmi of PUVA treated patients

Patient	Age (years)	Sex	Smoking	Disease	Ccmi Before treatment	CCmi After 20 session	CCmi After 40 session	CCmi After 60 session
1	16	M	No	Vitiligo	11.38	9.01	11.85	-
2	17	M	Yes	Vitiligo	9.96	9.59	8.78	11.39
3	18	F	No	Vitiligo	9.77	-	-	10.94
4	37	M	Yes	Vitiligo	11.23	-	-	11.08
5	28	F	No	Vitiligo	9.07	9.82	8.67	8.49
6	30	F	Yes	Vitiligo	10.39	11.33	11.84	11.36
7	52	F	No	Vitiligo	9.75	-	8.66	10.61
8	32	M	Yes	Vitiligo	9.81	8.02	-	9.79
9	33	F	No	Alopesi areata	9.36	8.95	8.86	8.83
10	13	M	No	Alopesi areata	8.15	8.84	9.12	9.05
11	16	F	No	Alopesi areata	9.04	-	10.95	-
12	13	F	No	Alopesi areata	10.49	11.91	-	-
13	16	F	No	Alopesi areata	8.24	8.81	9.94	-
4	23	F	No	Alopesi areata	9.59	8.67	9.71	11.09
15	26	F	No	Psoriasis	10.57	8.91	10.77	-
16	27	F	Yes	Psoriasis	9.30	9.34	10.13	-
17	18	M	No	Psoriasis	9.17	9.80	-	-
18	38	F	No	Psoriasis	11.87	11.68	-	-
19	28	F	No	Psoriasis	8.83	9.15	-	-
20	26	F	Yes	Psoriasis	9.88	10.52	-	-

Table II. CCmi of the patients before and after 20,40,60 sessions of PUVA treatment in comparison with their pretreatment values. Istatistical comparisons of CCmi before and after treatment have been done according to the Wilcoxon test.

Sessions of treatment	20 (n=16)	40 (n=12)	60 (n=10)
Mean dose ± SD(J/ cm^2)	57.67 ± 23.74	181.43 ± 46.41	299.56 ± 61.03
mg/kg of 8-MOP	0.6x20	0.6x40	0.6x60
CCmi (mean ± SD)			
Before treatment	9.76 ± 1.02	9.57 ± 0.93	9.65 ± 0.82
After treatment	9.65 ± 1.14	9.95 ± 1.19	10.27 ± 1.12
P	0.96	0.27	0.03

No significant correlation was found between age and CCmi at pretreatment (p=0.17).

DISCUSSION

Apoptosis considered to be the main action mechanism of PUVA therapy (13). Chromatin condensation occurs during a given step of apoptosis (14). In vitro PUVA treatment induces also apoptotic death of lymphocytes (4, 15). An increase of nuclear condensation is a typical feature of apoptosis (16).

UV irradiation (9) and chemicals treatment (8) of healthy cells cause some chromatin condensation in interphase nuclei .

In our previous study we have reported that treatment of patients with PUVA cause an increase in micronucleus (MN) frequencies in lymphocytes in vivo (7). However only dividing cells can express MN, non-dividing cell is unable to express its chromosome damage as MN. Therefore, MN assay can not be usable in non-dividing cell populations such as primary culture of brain and muscle tissue (17).

If one devise a simple method to test genotoxicity of a given agent on quiescent cells, this method may be applied also on non-dividing tissue/resting-cells population. Two observations have been reported on the chromatin condensation of the cells after the genotoxic-exposures (8,9). One of the observations was related with UV (9) and the other with a compound, cisplatin (8). The main constrains for the evaluation condensed chromatin (CC) were in the measurement step. In the last work (Jirsova and Mondys 1994), the CC evaluation was fully qualitative ordinal (normal, grained, worse grained, rough, coarse-grained, wrinkled) and not quantitative-numerical.

It has been demonstrated that a relationship exist between UVA doses and induced MN frequencies in PUVA treated patients (7).

To conclude, CCmi measurement in lymphocytes during PUVA therapy is eligible after 72 h of mitogen stimulation; but it's observed sensitivity begins at nearly 300 Joules/cm^2 of UVA and 0.6x60 mg/kg of 8-MOP. Long-term PUVA treatment causes a detectable CCi enhancement due to the DNA damages on its human users. CCi technique has been found less sensitive than the MN test for the gentoxicity assay on mitogen-

stimulated lymphocytes (in dividing cell populations). For the proper evaluation of the CCi measurement in interphase cells as a possible genotoxicity test, further investigation is needed.

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