Effect of Light Irradiation and Sex Hormones on Jurkat T Cells: 17β-Estradiol but Not Testosterone Enhances UVA-Induced Cytotoxicity in Jurkat Lymphocytes

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Abstract: In Eastern cultures, such as India, it is traditionally recommended that women but not men cover their heads while working in the scorching sun. The purpose of this pilot study was to determine whether there was any scientific basis for this cultural tradition. We examined the differential cytotoxic effects of ultraviolet A light (UVA) on an established T cell line treated with female and male sex hormones. CD4$^+$ Jurkat T cells were plated in 96 well plates at 2×10^6 cells/ml and treated with 17β-estradiol (EST) or testosterone (TE). These cells were irradiated by UVA light with an irradiance of 170 J/cm^2 for 15min at a distance of 6 cm from the surface of the 96-well plate. Controls included cells not treated with hormones or UVA. The effects of EST and TE were investigated between 1 and 20 ng/mL. Cytotoxicity by fluorescein-diacetate staining and COMET assay generating single strand DNA cleavage, tail length and tail moment measurements were examined. The effect of estrogen (5ng/mL) on apoptosis and its mediators was further studied using DNA laddering and western blotting for bcl-2 and p53. We found that EST alone, without UVA, enhanced Jurkat T cell survival. However, EST exhibited a dose-related cytotoxicity in the presence of UVA; up to 28% at 20 ng/ml. TE did not alter UVA-induced cytotoxicity. Since TE did not alter cell viability in the presence of UVA further damaging studies were not performed. COMET assay demonstrated the harmful effects of EST in the presence of UVA while EST without UVA had no significant effect on the nuclear damage. Apoptosis was not present as indicated by the absence of DNA laddering on agarose gel electrophoresis at 5ng/ml EST or TE ± UVA. Western blot showed that estrogen down regulated bcl-2 independently of UVA radiation while p53 was down regulated in the presence of UVA treatment. EST and TE have differential effects on UVA-induced cytotoxicity in Jurkat T-lymphocyte which suggested that women may be more susceptible to the harmful effects of solar irradiation than men.

Keywords: apoptosis, bcl-2, comet assay, cytotoxicity assay, estrogen, Jurkat T cells, p53, testosterone, ultraviolet light A.

Introduction

In Eastern cultures women but not men are advised to cover their heads while working in the scorching sun. To understand the potential underlying immune mechanism we chose T cells as the target cell for investigation. We assume that the difference between male and female is primarily due to the difference in sex hormone levels and the solar impact on the skin is mainly due to ultraviolet light A (UVA) light [1]. Thus, we formulated the hypothesis that sex hormones under the UVA radiation alter T cell responsiveness. To accomplish

our goals we studied the effect of estrogen and testosterone on established Jurkat CD4$^+$ T lymphocytes. First, the cytotoxicity of testosterone and 17β-estradiol ± UVA was determined. Then, the genotoxicity of UVA radiation on 17β-estradiol treated T cells was investigated along with the regulation of p53/bcl-2 pathway.

The tanning industry is rapidly growing in the United States. Currently, more than 1 million Americans use commercial tanning facilities every day [2]. The biggest categories of users are adolescents and young adults, especially women [1, 3]. Lamps currently used for recreational tanning emit UVA primarily or exclusively

[2]. UV light is known to induce skin cancers by causing DNA gene mutations and inducing immunosuppression [4]. Thus, it is important to study the direct effects of UVA radiation on immune T cells.

Sex hormones exert powerful effects in the susceptibility and progression of numerous human and experimental autoimmune diseases. This has been attributed to direct immunological effects of sex hormones that impact a clear gender dimorphism on the immune system. Globally, estrogens activate T-cell dependent humoral response thus contributing in the disease process while testosterone suppress T-cell immune responses and virtually always result in the suppression of disease expression [5]. Solar radiation has been proposed to play a detrimental role in the pathogenesis of cancer especially involving the skin and cervical cancer while a protective role in prostate cancer and colorectal cancer. Complex three way interactions between sex hormones, immune cells and UVA of the solar radiation appear to be involved in gender dimorphism of the immune system and immune system-related diseases such as cancer and autoimmunity.

The aim of this study was to determine whether treatment with female and male sex steroids altered the cytotoxic effects of UVA on Jurkat CD4$^+$ T lymphocytes and to determine related mechanism of action.

Materials and Methods

Reagents and Cell Lines

All reagents were purchased from Gibco (Grand Island, NY) unless otherwise stated. Hormones were obtained from Sigma (St Louis, MO). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). The human Jurkat T cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. The standard growth medium was prepared using RPMI 1640, 10% FBS and 1% antibiotic (100U/mL penicillin, and 100 μg/mL streptomycin,) and 2 mM L-glutamine.

Ultraviolet A Treatment

The T cells were placed in a 96-well plate with 100 μl in each well. Two nine well sets were used in each 96-well plate for each hormone concentration. One set was covered with aluminum foil as a dark control, and the other was irradiated with UVA light using a type B 100 W UVA lamp from UVP Inc. (Upland, CA). The lamp emitted a UVA light band near 365 nm with an irradiance of 170 J/cm^{2}per /h at a distance of 6 cm from the surface of the 96-well plate. The cells were irradiated for 15 minutes. Among the nine wells of each replicate set, six well were used for cell viability assays and the remaining three for the Comet assay. For cell viability assay the fluorescein diacetate was added directly to the wells and allowed to incubate for 35 minutes and read using a Fluroskan II microplate reader (Lab Systems, Helsinki, Finland) with an excitation wavelength of 485 nm, and an emission wavelength of 538 nm.

Cell Treatment for Acute Cytotoxicity

Cytotoxicity assay was carried out as previously described in our laboratory [6]. Briefly, cells were counted and resuspended at 20,000cells/100 μl (100 μl/well) in media. Aliquots of 100 μl of cell suspension were placed in wells of microtiter plates, and 100 μl of different concentrations of 17β-estradiol or testosterone (resulting in a final concentration of 0 or 1, 2, 5, 10 and 20ng/mL) were added to the respective wells used to treat the cells. The plates were incubated for 30 minutes at 37°C. After incubation, cells were centrifuged and washed twice with PBS. The PBS was removed and discarded and aliquots of 100 μL of fluorescein diacetate (10ng/mL) added. After 35 min incubation, the plates were read as described above.

Cell Treatment for Genotoxicity Assay

Cells were counted (10,000 cells/well) and re-suspended in media with 10% FBS. Aliquots of 100 μL of the cell suspension were placed in 96 well plates, treated with 100μl aliquot of either, media, 17β-estradiol or testosterone at 10ng/ml and incubated in a 5% CO_2 at 37°C for 72hrs. After incubation, the cells were centrifuged, washed with PBS, and re-suspended in 100 μL PBS. In a 2 mL tube, 20 μL of the cell suspension and 200 μL of melted agarose were mixed and 75 μL pipetted onto a pre-warmed slide. The slides were placed at 4°C for 15 min and then placed in chilled lysis buffer for 45 min. Slides were washed twice for 5 min with Tris-Borate-EDTA (TBE) and electrophoresed in a horizontal gel apparatus at 25 V for 10 min. Slides were placed in 70% ethanol for 10 min, removed, tapped, and placed in an alkaline solution (99 mL H_2O, 100 μL of 0.1 mM Na_2EDTA and 1 M NaOH) for 45 min. Slides were air dried for 2.5 hrs, stained with SYBR Green and allowed to set for 4 hrs at room temperature. The slides were viewed with an Olympus fluorescence microscope and analyzed using LAI's Comet Assay Analysis System software (Loates Associates, Inc. Westminster, MD).

DNA Laddering

Apoptosis was determined by electrophoresis of nucleosomal fragments using a standard procedure for precipitating cytosolic nucleic acid [7]. Briefly, 1 X 10^6 cells were pelleted (1200 X g, 5min) and lysed for 15 min (250μl, 0.4% Triton-X, 20mM Tris, 0.4mM Na_2EDTA) at 4°C. Nuclei were then pelleted (13,000 x g, 5 min, 4°C) and the supernatant was transferred to a clean microfuge tube. Nucleosomal fragments were precipitated overnight with an equal volume of isopropanol after adjusting to 0.5 M NaCl. The pellet which represents precipitated cytosolic DNA, was washed twice in 70% ethanol, dried briefly, and re-suspended in 40 μL TE (10 mM Tris-HCl, 1 mM Na_2EDTA) with 1 mg/ml DNase-free RNase. Results were identical to those in which total DNA was prepared, but this modification facilitates resuspension of the DNA fragments which are transported from the nuclear location to the cytosol. A total of 15 μl was

electrophoresed on a 1.8% agarose gel and stained with ethidium bromide for visualization. A representative experiment is shown in Figure 7. The picture of the gel in Figure 7 is the consequence of one of three experiments.

Western Blotting

The procedure utilized was as outlined in the manuscript by Jenkins et al. [7]. Briefly, cells were pelleted, washed with PBS. The PBS was discarded and cells were lysed in 100 µl of protein lysis buffer containing protease inhibitors. The lysed cellular protein solution was placed on ice. SDS-PAGE (12.5%) gels were loaded with sample and electorphoresed at 125 V. After electrophoresis, the gel was transferred to a nitocellulose membrane (Sigma, St. Louis, MO) overnight at 4^OC. After transfer, the membrane was placed in blocking solution (5% milk) on a shaker for 60 min at RT, washed with PBS-0.05% Tween (PBST). This was incubated with primary antibody (p53 or bcl-2 [mouse anti-human monoclonal antibodies]) diluted in 1% milk/PBST solution on a shaker 1 hr at RT, and washed 3 times for 5 min in PBST. The membrane was incubated with secondary antibody conjugate horse radish peroxidase (goat anti-mouse IgG monoclonal) for 60 min at RT on a shaker and washed 30 min in PBST. The chemiluminescent substrate was added for 1 min prior to the membrane being exposed to X-ray film.

Statistical Analysis

Cytotoxicity and comet (n=70) assay measurements were reported as means ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA for multiple samples or by *Student's-t*-testing with matched pairing if appropriate. For statistical analysis F-statistic ANOVA was applied to determine if there were significant differences in genotoxicity with regard to hormone and UVA exposure. Differences were considered significant at P values ≤0.05.

Effect of Estrogen ± UVA on JKT cells
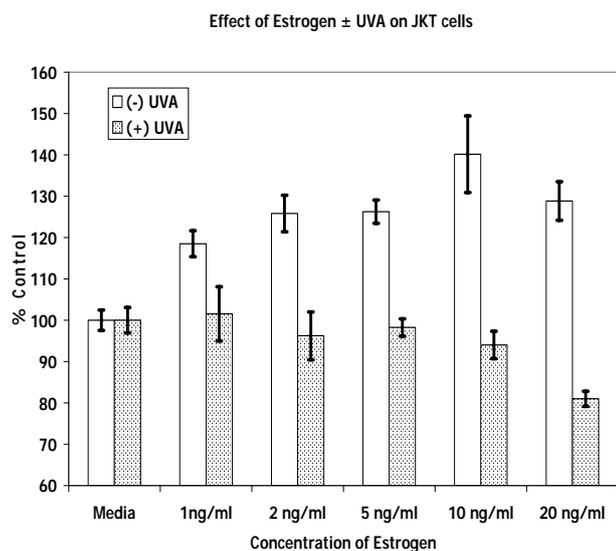
Figure 1: Cell viability of Jurkat T cells at different concentrations of estrogen ± UVA.

Results

The viabilities were tested in the concentration range 0 to 20ng/mL. Figure 1 and Figure 2 depicts the viability of Jurkat T cells with estrogen and testosterone ± UVA light. Figure 1 showed that estrogen alone without UVA enhanced T cell survival. Estrogen in the absence of UVA increases cell viability with 5ng/ml, 10ng/mL and 20ng/ml exhibiting 126%, 140% and 128%. However, in the presence of UVA light and estrogen, the survival decreases to 78% at 20ng/ml. The means of survival for estrogen treated T lymphocytes ± UVA light are statistically significant (2ng/ml and 5ng/ml, $p<0.05$ and 10ng/ml and 20ng/ml, $p <0.01$).

Effect of UVA on JKT using Testosterone
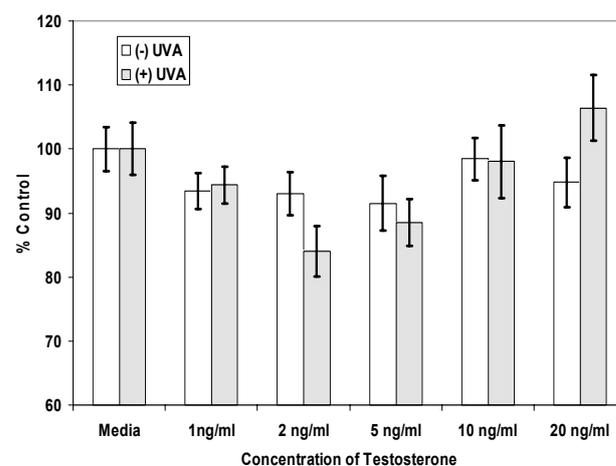
Figure 2: Cell viability of Jurkat T cells at different concentrations of testosterone ± UVA.

Figure 2 shows that in the absence of UVA the cell viability varies from 92-97% while in the presence of UVA light there was a transient decrease in viability to 85% at 2 ng/mL but was steady at 5 to 20 ng/ml from 88 to 108%. The differences in means of survival with testosterone ± UVA did not reach statistical significance. Testosterone (20 ng/ml) + UVA partially improve cell viability.

Figure 3 shows comet assay picture of cells ± UVA with estrogen at 5ng/mL. The nuclear DNA of untreated cells was round while estrogen + UVA were severely dispersed and fragmented. The percentages of DNA cleavage was approximately 8.5, 4.1 and 7% fragmentation (Figure 4), and the lengths of comet tail varied 37, 34 and 27% (Figure 5) while tail moment varied from 9.1, 2.7 and 5.8% (Figure 6) at 5, 10 and 20 ng/ml estrogen concentrations respectively. There are several ways to measure the severity of DNA fragmentation. In our study the variables studied were the percent of DNA fragmentation (percent of DNA in the Comet tail versus total DNA), tail moment and the length of the comet tail. Tail Length is the distance between the head and the last DNA fragment and Tail Moment is the product of % DNA and tail length mathematically written as (%DNA x Tail Length). The higher the percent of DNA fragments, the more severe is the damage. Similarly, the longer the comet tail, the smaller is the DNA fragment, the more severe is the damage.

Comet Assay
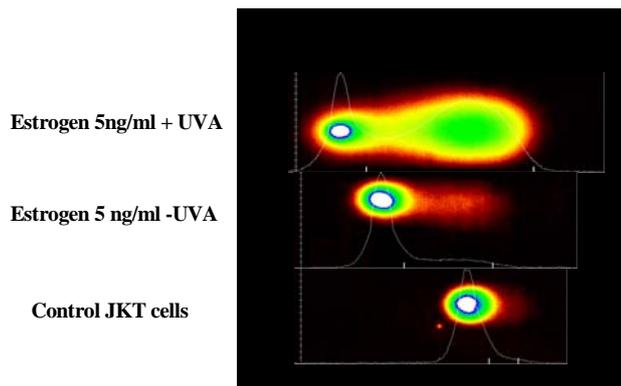
Figure 3: Comparison of Comet assay of Jurkat T cells untreated as controls and cells treated with estrogen at 5 ng/ml ± UVA.
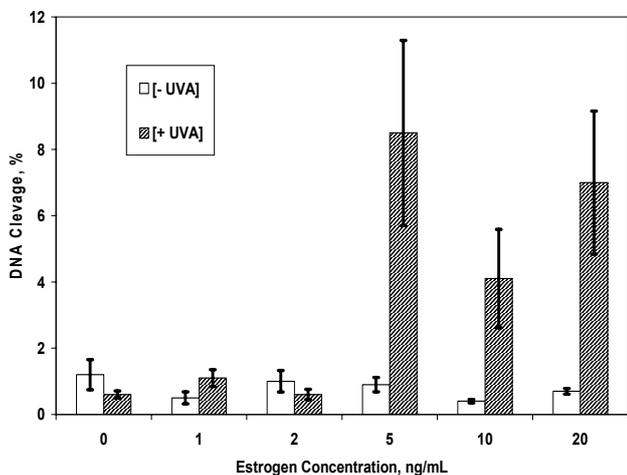
Figure 4: Comet assay of Jurkat T cells treated with estrogen ± UVA where Y-axis is the percentage of DNA Cleavage and x-axis is the different concentrations of estrogen used in comparison with controls which had no estrogen.
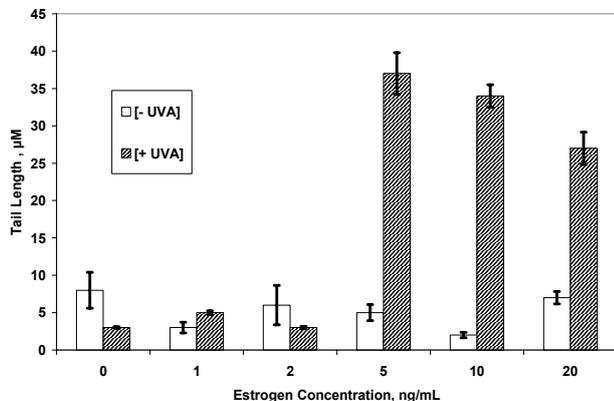
Figure 5: Comet assay of Jurkat T cells treated with estrogen ± UVA where Y-axis is the Tail Length (μm) and x-axis is the different concentrations of estrogen used in comparison with controls which had no estrogen.
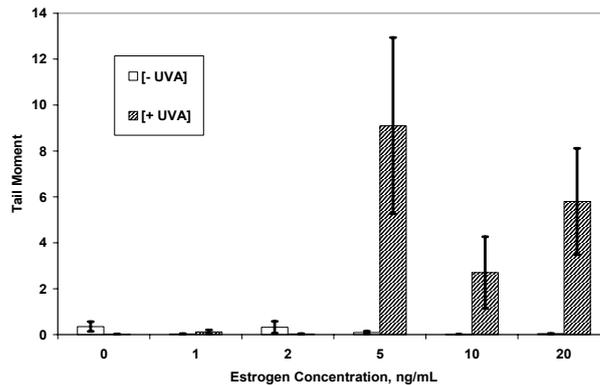
Figure 6: Comet assay of T cells treated with estrogen ± UVA where Y-axis is the Moment and x-axis is the different concentrations of estrogen used in comparison with controls which had no estrogen.

DNA laddering was determined as shown in Figure 7. This shows that UVA exposure of estrogen treated cells does not alter DNA laddering of T cells.

DNA Laddering
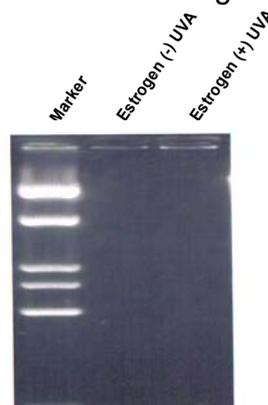
Figure 7: DNA laddering of cells treated with estrogen ± UVA.

Western blot (Figure 8) shows that bcl-2 was significantly and equally down regulated by estrogen and that ± UVA radiation had no effect. p53 is absent from the cells that were not treated with estrogen but was present when estrogen was added. UVA treatment decreased p53 expression.
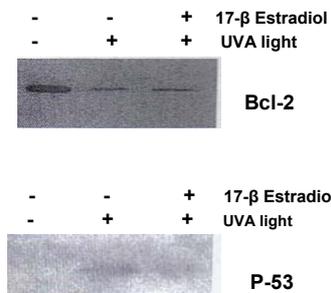
Western Blot of Bcl-2 and p-53

Figure 8: Expression of bcl-2 and p53 by western blot of cells treated with estrogen in the presence and absence of ultraviolet A

Discussion

The sun emits a wide variety of electromagnetic radiation, including infrared, visible, ultraviolet A (UVA; 320 to 400 nm), ultraviolet B (UVB; 290 to 320 nm), and ultraviolet C (UVC; 10 to 290 nm) [3]. The only ultraviolet radiation wavelengths that reach the Earth's surface are UVA and UVB. UVA rays pass deeper into the skin. However, its predominance in the solar energy reaching the Earth's surface (tenfold to one hundredfold more than UVB) permits UVA to play a far more important role in contributing to the harmful effects of sun exposure than previously suspected. Thus, the protection from UVA has profound implications on public health worldwide.

Studies have suggested that UV exposure can negatively affect the body's immune system by interfering with the production of disease-fighting T cells and therefore lower the body's natural defences against infection [3]. UV exposure modifies local and systemic immune responses by activating the T cell suppressor pathway. It has been suggested that prolonged exposure to sunlight may induce systemic or local immune alterations, which may facilitate the development of non-Hodgkin's lymphoma [8]. The effects of prolonged sunlight exposure on peripheral blood cells showed an increase in cells expressing the interleukin-2 receptor (IL-2R) and, more specifically, an increase in the T cells expressing IL-2R and HLA-DR antigens [9]. These findings suggest that prolonged intense exposure to sunlight may be associated with immunostimulation, rather than immunosuppression [9]. Thus, it is important to study directly the effect of UVA radiation on immune T cells.

The sun's UVA rays are also linked with skin cancer and with the wrinkling that comes from sun exposure. UVA radiation primarily mediates singlet-oxygen damage triggering immediate pre-programmed cell apoptosis (T < 20 min) by immediately opening the cyclosporine A-sensitive ("S" site) mitochondrial megapore, while super oxide anions initiate another cyclosporine A-insensitive ("P" site) final apoptotic pathway [10]. This would imply that longer than 20 minute exposure of UVA radiation leads to total apoptosis which can be detected by DNA laddering.

Sun exposure in some cases can be helpful. UVB rays are the sun's ultraviolet rays linked with tanning and burning. Exposure to sunlight reduces the risk of prostate cancer [11]. Prognosis of breast, colon, ovary, and prostate as well as non-Hodgkin lymphoma may be related to synthesis of vitamin D(3) which is dependent on the degree of UVB exposure [12-15].

The dilemma that sun exposure on one side is dangerous and on the other hand helpful can be explained by understanding the difference between UVA and UVB radiation. UVA radiation is harmful while UVB radiation which is linked with Vitamin D synthesis is beneficial [15, 16].

Despite the harmful levels of solar UV radiation, mechanisms have evolved to protect cells and to repair damaged molecules. The cell component most vulnerable to injury is nuclear DNA. A number of different DNA repair mechanisms have been established [17]; the best known being photo reactivation, excision repair [18], post replication repair and SOS repair [17]. It seems that 15 minute UVA exposure of T cells treated with estrogen is not sufficient to give a permanent damage as there was no apoptosis in the DNA laddering assay.

Effect of UVA light on Jurkat T cells in combination with azulene [19] and hydroxypyrene [20] has been previously studied. No study has yet appeared in the literature which studies the effect of UVA irradiation on estrogen- and testosterone-treated Jurkat T cells.

A variety of evidence exists that hormones are thought to exert modulatory effects on immune function [7]. It is well documented that sex hormones testosterone and 17-ß-estradiol play an important role in autoimmunity, pregnancy, menopause and prostate cancer [5, 7]. Direct hormone-specific effects on Jurkat CD4 + T lymphocytes have been studied previously by Jenkins et al. and McMurray et al [7, 21]. 17β-estradiol has been shown to inhibit Jurkat T cell proliferation, stimulate accumulation of cells in S and G2/M phases of the cell cycle, and induce apoptosis over 72 h in a dose-dependent manner [7]. Bcl-2 is a marker for uncontrolled cell growth [22, 23]. Bcl-2 protein and mRNA were also reduced in estrogen treated Jurkat T lymphocytes [7]. Additionally, bcl-2 protein levels were suppressed in association with estrogen-induced apoptosis. In our hands also bcl-2 protein was suppressed in the presence of estrogen and UVA radiation exerted no influence on its expression.

Testosterone maintains lean body mass, bone density, skin elasticity and libido. It also modulates many physiological processes, including cytokines and immune T cells [24], in addition to being a precursor for the formation of estrogen [25]. Estrogen (17β-estradiol) modulates the course of both the menstrual cycle and menopause, so imbalances are directly linked to symptoms such as weight gain, headaches, premenstrual syndrome, mood swings, and abdominal cramps. Estrogen deficiency at menopause increases a woman's risk of bone loss and osteoporosis. With the onset of menopause, however, decreased synthesis of 17β-estradiol is accompanied by an increased incidence of cardiovascular disorders and accelerated progression of renal diseases [26].

Our data shows that testosterone >5 ng/ml improves lymphocyte viability while UVA exposure does not induce significant cytotoxicty. On the other hand, estrogen improves viability in a dose-response fashion but in the presence of UVA radiation it is cytotoxic. This implies that females are more prone to lymphocyte damage when exposed to sun specifically by UVA radiation as they have estrogen in the blood stream and lymphocytes in the skin.

Several pathways have been described for apoptosis in different systems, but they all converge with activation of the protease caspase 3 and subsequently of endonuclease activity that results in fragmentation of nuclear DNA [27-32]. Experimentally, this DNA fragmentation has been demonstrated in model systems as a "ladder" following electrophoretic separation of DNA extracts prepared from the cell population. This method lacks sensitivity because the proportion of apoptotic cells in the population must be large to be detected. A method has been developed to detect DNA damage in single cells called the "comet" or single cell gel electrophoresis assay that can detect effects in subpopulations of cells [33]. Since UVA radiation in the

presence of estrogen affected cell viability, we investigated the cytotoxic phenomenon by studying DNA damage using the comet assay. The comet assay revealed that DNA cleavage, tail moment and tail length were all increased in the presence of UVA radiation at a concentration >5 ng/ml of estrogen. Thus, our data shows that for a 15 minute exposure of UVA + estrogen, at the individual cell level, is damaging but not a sufficient number of cells are damaged at the population level to show DNA laddering.

DNA damage caused by UV radiation, which if left un-repaired, results in molecular alterations in the skin and blood which eventually lead to skin mutations found in skin cancer. Skin biopsies, as well as blood samples when taken after a single UV exposure were tested for p-53. Specifically, the study analyzed the amount of p53 protein in the skin and blood. Within 24 hours of the first UV exposure, p53 protein was present in all layers of the epidermis [34]. The increased presence of p53 protein in the skin signifies that the body is responding to the cell damage due to UV exposure. p53 is a protein which allows cells to slow down their reproduction process so that damage from UV radiation can be repaired [35-36]. Though the body is trying to repair the damage, there is the risk of a mistake in the repair process, which increases as the number of altered cells multiply. If there is a 'miss' in the cell repair process, subsequent replication of the altered cell may yield a clone of abnormal cells which may eventually appear as a skin cancer. The p53 tumor suppressor plays a key role in protection from the effects of different physiological stresses (DNA damage, hypoxia, etc.), and loss of its activity has dire consequences, such as cancer [36, 37]. In our study we found that estrogen up-regulates the expression of p53, but that p53 is down- regulated in the presence of UVA. Thus, the absence of p53 exhibits a decrease in defence mechanisms of T cells in the presence of estrogen when exposed to 15 minutes of UVA radiation. The mechanism of action of UVA radiation is depicted in Figure 9.

Mechanism of action of Estrogen
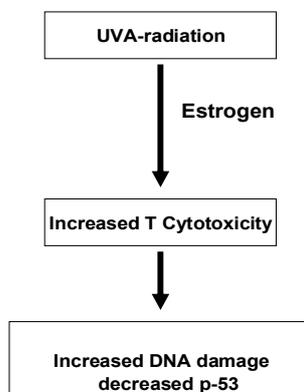
Figure 9: This figure represents the mechanism of action of estrogen. Under the influence of UVA radiation estrogen causes increased T cytotoxicity which results in increased DNA damage and decreased p53.

Most autoimmune diseases have been associated with the female gender, e.g. systemic lupus erythematosus (SLE). Female sex hormones, primarily estrogen and progesterone, have been implicated in this predisposition of the female gender to autoimmunity. Compared to the male sex steroid testosterone, these female hormones have been shown to have different effects on lymphocytes with respect to activation, proliferation, apoptosis and cytokine production [7, 21]. These differences may be responsible for the differences in the prevalence of autoimmunity and the immune hyper-responsiveness seen in women [7, 21, 38]. Interestingly, the autoimmune disease SLE commonly exhibits photosensitivity and skin rashes. Clinically uninvolved skin characteristically exhibits histologic evidence of immune reactivity. Photosensitivity is included as one of the eleven clinical diagnostic criteria for lupus (38). Furthermore, SLE patients may exhibit a flare of their systemic autoimmune disease after sun exposure (38). Enhanced DNA cleavage and reduced viability of lymphocytes with UVA, presumably an anti-immune effect, suggests estrogen might not play a role in cutaneous lupus, suggesting other female hormones, such as progesterone, may be the culprit. This is in agreement with recent reports suggesting that progesterone rather than estrogen are responsible for the immune hyper responsiveness in SLE [38]. Thus, the solar flares seen in females with SLE are not due to estrogen and therefore not related to the findings in this study.

In 2004, more than one million cases of non-melanoma skin cancers are expected to be diagnosed in the United States [39]. An obvious question is whether men or women are at higher risk in view of the findings in this study. Unlike the case with cutaneous SLE, we are unable to interpret our findings with respect to the gender predisposition to skin cancer for the following reasons: basal cell and squamous cell carcinoma (since they are highly curable) are not traditionally included in overall cancer statistics, and no information is available regarding the proportions of women or men who intentionally tan and develop skin cancer. With respect to the issue of skin cancer in India, and the potential preventive effects of covering up in the scorching sun, it has not been possible to obtain appropriate statistics from India regarding the incidence of cancer in women and men.

In conclusion, we have shown in our study that estrogen adversely affects T cells under the influence of UVA. Secondly, the UVA induced cytotoxicity caused damage at the individual cell level but the damage is probably repaired by the cellular processes as shown by the laddering assay. Western blot analysis showed that estrogen down regulated p-53 in the presence of UVA treatment. These studies suggest that the mere presence of estrogen may modulate the activity of T cells. It is possible that prolonged duration of UVA light may be very harmful to females and may be protective to males. This study provides a scientific basis for the cultural tradition in India in which females not males cover their heads with usually a cloth while in the scorching sun.

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