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Administration of Caffeine Cream Increased Apoptotic Cells and Decreased Phosphorylated ATR (Thr 1989) Expression in Ultraviolet B Exposed Skin Mice (*Mus musculus*)

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ABSTRACT

Background: Chronic exposure of human skin to ultraviolet B (UVB) affects the structure and function of the skin, and causing skin aging. Caffeine is hypothesized to inhibit the skin aging process through the inhibition mechanism of the Ataxia Telangiectasia and Rad3-Related–Checkpoint Kinase 1 (ATR-Chk1) pathway. This study aimed to prove that caffeine cream can increase the apoptotic cells and decrease the expression of phosphorylated ATR (Thr 1989) in the skin of male mice (*Mus musculus*) exposed to UVB.

Methods: This study used a randomized post-test only control group design. The subject used in this study was 36 mice and divided into two groups. The control group was treated with emulgide cream as a placebo and the treatment group was treated with cream containing 4.8% of caffeine. Both groups were exposed to UVB at a dose of 343 mJ/cm^2 for 8 weeks. Skin biopsy from the central area of the back obtained after the last session and followed by an examination of apoptotic cells of epidermis using H&E staining and phosphorylated ATR (Thr 1989) expression in epidermis using immunohistochemistry.

Results: It showed that the mean number of apoptotic cells in the treatment group was higher (2.15±0.43 cells/hpf) than the control group (0.81±0.55 cells/hpf) ($P<0.001$). The mean phosphorylated ATR (Thr 1989) expression in the treatment group was lower (8.03±3.91%) than the control group (23.62±4.39%) ($P<0.001$).

Conclusion: The administration of caffeine cream can increase apoptosis and decrease phosphorylated ATR (Thr 1989) expression in the UVB-exposed male mice (*Mus musculus*) skin epidermal cells.

Keywords: UVB, skin, caffeine, apoptosis, phosphorylated ATR.

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INTRODUCTION

Chronic exposure to ultraviolet affects the structure and function of the skin. Exposure to UVB radiation is a major risk factor in causing skin aging or known as photoaging.[1,2] Ultraviolet radiation categorized as a complete carcinogen because of its mutagenic effect and act as a tumor initiator.[3] The literature showed that the incidence of melanoma is higher in areas closest to the equator, especially Caucasians, which have lower skin melanin pigment and therefore are most sensitive to UV.[4] Studies from the Norwegian Cancer Registry reported that a 2-2.5-fold increase in melanoma risk is associated with a 10° decrease in latitude.[4]

The mutagenic potential of ultraviolet radiation is a consequence of DNA damage namely cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone 6-4 photoproducts (6-4PPs). Human skin exposed to the sun for a long duration can cause photodamage-related skin changes including formation of wrinkles, solar elastosis, degradation of macromolecules matrix in the dermis and increase risk of direct mutations in DNA.[5,6]

In early stage of DNA Damage Response (DDR), an important pathway that is activated in response to ultraviolet is phosphoinositol-3-kinase (PI3K) related protein kinase (PIKKs) which includes ATR (Ataxia Telangiectasia and Rad3-Related). At several points of ATR phosphorylation, only Thr 1989 is important in amplifying ATR activation.[4] With the tropical climate in Indonesia, where people are often exposed to sun light, the photodamage process includes DNA Damage Response (DDR) is very likely to take place significantly. Topically administered caffeine has shown significant results in preventing UVB-induced photodamage, but the mechanism is not fully understood. Caffeine is thought to be an inhibitor of ATR and can override the replication checkpoint function which causes cells to go to Premature Chromatin Condensation (PCC) and as a result, apoptosis in defective cells. In

2009, Heffernan *et al* used cell line in vitro study to detect the role of caffeine in the protective function of epidermal cells after UVB exposure. They found that caffeine administered to the cell line decreased the expression of Ser-Thr kinase checkpoint kinase-1 (Chk1) as a substrate of ATR, then it could reduce the number of cells with DNA damage.[8]

Several studies of caffeine as skin protection for ultraviolet radiation have been published.[6,8,9] However, study to determine proximal markers of the ultraviolet-induced ATR-Chk1 pathways in DNA damage response which induced by ultraviolet radiation has not been carried out, therefore another study is needed to determine role of caffeine in inhibiting the photodamage process.

This study aimed to prove that giving a caffeine cream can increase apoptosis and decrease phosphorylated ATR (Thr 1989) as a proximal marker of the ATR-Chk1 pathway in an animal study (*in vivo*) of male mouse (*Mus musculus*) skin epidermal cells exposed to ultraviolet B radiation.

METHODS

This study used a randomized post-test only controlled group design. This research was conducted in the Integrated Laboratory Unit of Udayana University Bali and Sentra Pathology Laboratory Bali.

Experimental Animal

This study used 36 male mice (*Mus musculus*) available from the animal unit of Medical Faculty, Udayana University and has received approval from the Ethics Committee of the Udayana University. The sample inclusion criteria in this research were healthy, 6-9 weeks old, male mice with 20-30 grams of weight. The mice were divided randomly into 2 groups. Control group was treated with basic cream (emulgide cream) and UVB exposure, while treatment group was treated with 4.8% caffeine cream and UVB exposure. A day before the treatment, the mice from all groups were being shaved on the back skin in 2x2 cm size. The mice in the control and treatment group were irradiated with UVB at a dose of 343 mJ/cm^2. Hereafter, the mice in the treatment group were treated with 4.8% caffeine cream and the mice in the control group were treated with basic (emulgide) cream as a placebo. The treatment was repeated 3 times a week for 8 weeks (24 sessions in total). The mice either from the control or treatment group were treated by UVB light exposure using Philips PL-S 9W/01/2P (Poland) lamps 3 times a week (Monday, Wednesday, and Friday) for 8 weeks every 09.00 am WITA by the dose of 343 mJ/cm^2. Distance between UVB exposed to the mice was measured by using a UV light meter YK-35 UV.

Skin biopsy was performed after the last treatment session (the 24th session). Tissues were fixed immediately in 10% phosphate buffered formalin and then processed for H&E to determine apoptotic cells and immunohistochemistry to determine expression of phosphorylated ATR (Thr 1989).

Examination of Apoptotic Cells

Apoptosis was counting in the number of epidermis and was seen in H&E staining as cell with eosinophilic cytoplasm and dense, hyperchromatic and small nuclei. The analysis of apoptotic cells in the epidermis was conducted using Optilab Pro Viewer 2.2 (Miconos, Indonesia) connected to the microscope Olympus CX21 (Olympus, Japan). Three epidermis foci were captured in 400x magnification and the images were saved in the form of JPEG. The calculation was done by using Image Raster software and the mean of the counting results was taken. The amount of apoptotic cells expressed in the number of cells/hpf.

Examination of Phosphorylated ATR (Thr 1989)

Phosphorylated ATR (Thr 1989) is the expression of active ATR protein in epidermis evaluated by immunohistochemistry method using Phospo-ATR antibody (GeneTex) with 1: 200 dilution which was seen as brown stained nucleus in epidermal cells. The calculation was done with the percentage of positive cells divided by the number of all epidermal cells in one field of view with 400x magnification. The analysis was conducted using Optilab Pro Viewer 2.2 (Miconos, Indonesia) connected to the microscope Olympus CX21 (Olympus, Japan). Three epidermis foci were captured in 400x magnification and the images were saved in the form of JPEG. The counting was done by using Image Raster software and the mean of the counting results was taken. ATR expression was stated in percentage (%).

Statistical Analysis

The data were processed using the IBM SPSS Statistic program. The normality test was performed using the Shapiro-Wilk test. The comparison test was carried out using a non-parametric test, Mann Whitney U. A p-value of less than 0.05 ($p < 0.05$) was considered significant.

RESULTS

The descriptive results of the table showed that the mean levels of the apoptotic cells in the treatment group were higher compared to the control group. While on the levels of phosphorylated ATR (Thr 1989), the treatment group has a lower mean compared to the control group. The results of homogeneity tests showed that the data is homogeneous. The results of the normality test showed an abnormal distribution so that the comparison test was carried out using a non-parametric test, Mann Whitney U.

Table 1. Descriptive Data of Apoptotic Cells and Phosphorylated ATR (Thr 1989).

Variable	Subject group	N	Average ± SD	Median (min-max)
Apoptotic cells (cells/large field of view)	Control	18	0.81±0.55	0.66 (0.00-1.67)
	Treatment	18	2.15±0.43	2.16 (1.67-3.00)
Phosphorylated ATR (Thr 1989) (%)	Control	18	23.62±4.39	23.30 (18.30-7.70)
	Treatment	18	8.03±3.91	5.70 (4.30-16.00)

N = number of samples; SD = standard deviation

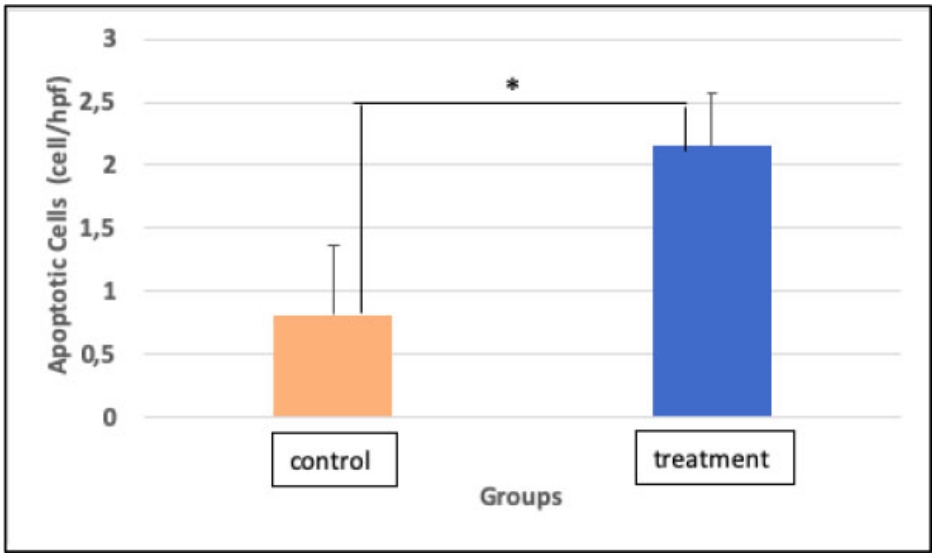

Figure 1. Apoptotic cells in treatment group was significantly higher than control group (* p<0.05).

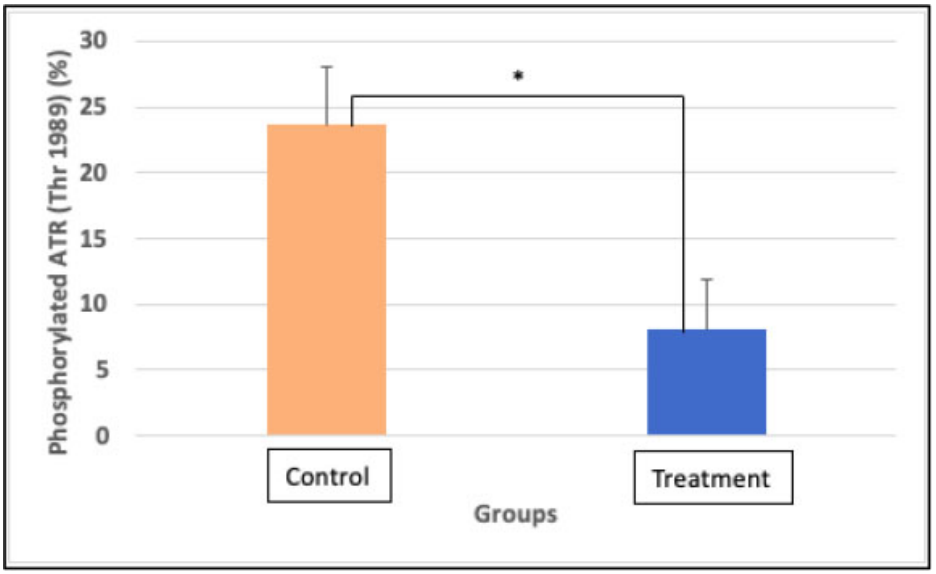

Figure 2. Phosphorylated ATR (Thr 1989) amount in treatment group was significantly lower than control group (* p<0.05).

The comparative test showed that the mean level of the apoptosis in the control group was 0.81±0.55 cells/hpf and in the treatment group was 2.15±0.43 cells/hpf. Analysis of significance using the Mann Whitney U test showed p value <0.001, which means that there were significant differences between two groups. These results indicated that the mean levels of the apoptotic cells in the treatment group were significantly higher than the control group. The difference in the mean number of apoptosis in the control group and treatment group can be observed in Figure 1.

The results of the comparative test analysis showed that the mean amount of phosphorylated ATR (Thr 1989) in the control group was 23.62±4.39% and in the treatment group was 8.03±3.91%. Analysis of significance using the Mann Whitney U test showed a p value <0.001, which indicated a significant difference between the two groups. This shows that the mean amount of phosphorylated ATR (Thr 1989) in the treatment group is significantly lower than the control group. The difference in the mean of phosphorylated ATR (Thr 1989) expression in control group and treatment group can be observed in Figure 2.

The histological result of the control and treatment group is shown in Fig. 3. In H&E staining, it was found that the apoptotic epidermal cells amount was found more in the treatment group compared to the control group. While in the immunohistochemistry staining, the epidermal cells that expressed phosphorylated ATR (Thr 1989) in the treatment group is fewer than the control group.

DISCUSSION

This study is an experimental study using male mice aged 6-9 weeks. The selection of mice as experimental animals because mice have the same skin structure as humans and at 6-9 weeks of age the skin structure of mice is the same as that of young adult humans. The male sex was chosen because it was found that male mice experienced photodamage more significantly after being exposed to UVB rays.[10,11]

The study showed that the mean levels of the apoptotic cells in the treatment group which was given 4.8% caffeine cream was higher than the control group with repeated exposure to UVB rays. The dose of UVB exposure used is 3 times a week for a total 8 weeks (24 sessions) of 343 mJ/cm^2, adjusting to previous research that at that dose can cause epidermal hyperplasia. Increased and repeated exposure to UVB rays can lead to Epidermal Growth Factor Receptor (EGFR) activation which will cause skin proliferation and suppress apoptosis with the end result of skin thickening or epidermal hyperplasia.[10]

Exposure to UVB rays can cause direct DNA damage by the formation of cyclobutene pyrimidine dimers (CPDs) and pyrimidine-pyrimidone 6-4 photoproducts (6-4PPs). Human skin exposed to the sun for a prolonged period time can experience photodamage, including an increased risk of direct mutations in DNA. The increased number of apoptotic cells after administration of caffeine cream was to eradicate cancer cells caused by UVB radiation.[12,13]

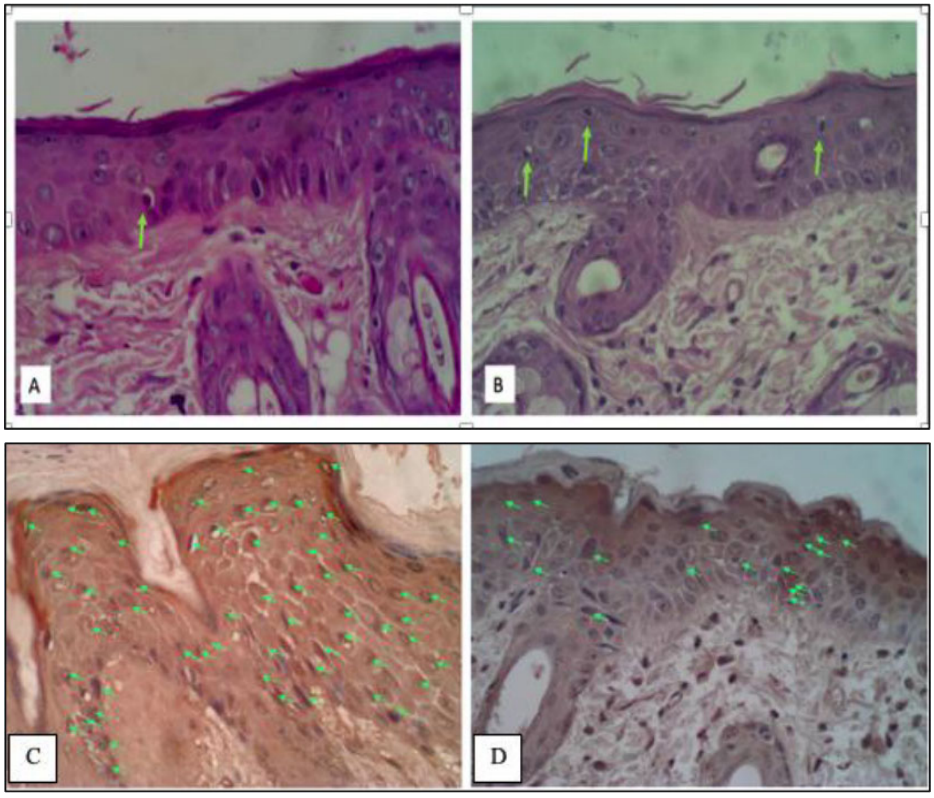

Figure 3. Apoptotic cells and phosphorylated ATR (Thr 1989) expression in mice (*Mus musculus*) skin using H&E and immunohistochemistry with 400x magnification. Apoptosis of epidermal cells was found more in the treatment group (B) than the control group (A) (green arrows). Epidermis that expressed phosphorylated ATR (Thr 1989) was fewer in the treatment group (D) than the control group (C) (green arrows).

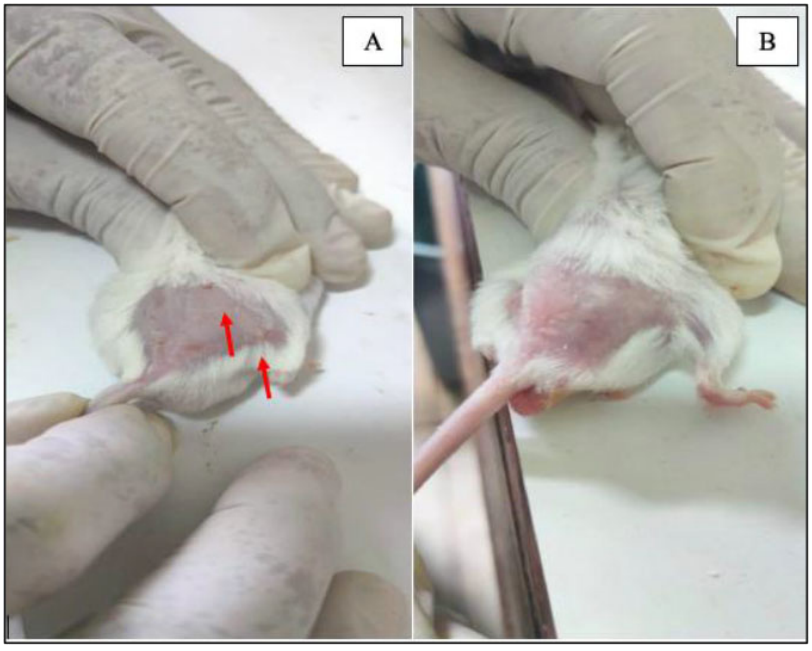

Figure 4. Skin appearance of mice (*Mus musculus*) after treatment. The control group (A) showed more thickening skin that indicated a photodamage (red arrows) compared to the treatment group (B).

The main target of caffeine cream on the skin is Ataxia-Telangiectasia and Rad-3-related (ATR) which is a protein kinase involved in various stressors including DNA damage due to UVB exposure. Caffeine cream is an inhibitor of ATR so that it can cause cells to go to premature chromatin condensation (PCC) and then apoptosis occurs. Apoptotic cells in the epidermis or sunburn cells (SBC) are believed to protect and act as resistance against the accumulation of genetic damage and the progression of skin cancer.[14] The mean levels of the phosphorylated ATR (Thr 1989) expression in this study were significantly lower in the treatment group than in the control group with repeated exposure to ultraviolet B ($p<0.001$). This shows that the caffeine cream effective in reducing the expressions of phosphorylated ATR (Thr 1989) on the skin of mice exposed to UVB rays.

ATR is a large group of kinases with a sequence similar to the lipid kinases of the phosphatidylinositol-3-kinase (PI3K) family. Signaling from ATR-Chk1 is activated when DNA replication is inhibited, for example by ultraviolet light. When replication stops, DNA polymerase detaches from the helicase, then producing single strand DNA (ssDNA) channels that are rapidly coated by Replication Protein A (RPA). ATR-mediated phosphorylation of Chk1 after UVB irradiation results in decreased levels and function of cyclin B1 which subsequently slows mitotic entry and prevents premature chromatin condensation. Inhibition of ATR by caffeine in UVB exposed cells results in a premature increase of cyclin B1 and premature chromatin condensation followed by apoptosis.[15]

In DNA Damage Response (DDR) and especially in UVB-induced damage, primarily activating the ATR pathway which then phosphorylates the underlying pathways. Caffeine inhibits ATR activity but not directly to Chk1. Several studies used phosphorylation of Chk1 as a marker to determine the ATR-Chk1 pathway. This is due to the limitations of proximal biomarkers to detect the previous pathway. However, it was found that ATR phosphorylation at Thr 1989 sites is a good biomarker in detecting active ATR. In its

activation, ATR undergoes a change to hyperphosphorylated state with various phosphorylation sites, and only Thr 1989 is crucial in amplifying the activation.[16]

This study proves that unlike chronological aging which is mainly determined by the predisposition of each individual, skin aging caused by ultraviolet rays, especially UVB depends mainly on the degree of sun exposure. Clinical signs of skin aging due to ultraviolet radiation (photoaging) include wrinkles, pigmentation disorders, skin atrophy, telangiectasia, solar elastosis, precancerous lesions, skin cancer, and melanoma. Ultraviolet radiation can induce various photoproducts, such as cyclobutene pyrimidine dimers (CPDs) and pyrimidine pyrimidone 6-4 photoproducts (6-4PPs) and there is some evidence that CPDs is a pre-carcinogenic lesion.[2,17]

With increased apoptotic cells and decreased expression of phosphorylated ATR (Thr 1989) by giving caffeine cream to the skin of mice exposed to UVB rays, it is hoped that this study can be a reference that caffeine cream can prevent photodamage caused by UVB exposure.

CONCLUSIONS

It can be concluded that the caffeine cream can increase the apoptotic cells and decrease the expression of phosphorylated ATR (Thr 1989) in male mice (*Mus musculus*) skin exposed to ultraviolet B. It is necessary to explore the potential side effect of caffeine cream on the skin at certain concentrations. Also, further research needs to be done to identify the optimal concentration of caffeine cream in preventing photodamage and it is necessary to develop the results obtained from this study as a comparison to other medications in the prevention of photodamage of the skin.

ETHIC APPROVAL

Ethics approval was obtained by the animal ethics committes, Faculty of Veterinary Medicine, Udayana University, before this research was carried out with ethical clearance reference number No.83/UN14.2.9/PT.01.04/2020.

CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

AUTHOR CONTRIBUTION

All authors equally contribute to the study from the research concepts, data acquisitions, data analysis, statistical analyses, revising the paper, until reporting the study results through publication.

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