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### Original Article

## Protective effects of daily topical cocoa extract to the expression of 8-OHdG and PCNA on UVB-exposed albino mice

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#### ABSTRACT

**Background.** Chronic impact caused by repeated exposure to low energy levels of ultraviolet B (UVB), are in the form of precancerous, skin cancer and premature aging. 8-hydroxy-2-deoxyguanosine (8-OHdG) and proliferating cell nuclear antigen (PCNA) known as biomarker for UVB-induced DNA damage. Antioxidant are used to protect the skin from UVB-induced DNA damage, and cocoa is one of them due to their polyphenol content. **Aims.** Examine the protective effects of daily topical cocoa on UVB-exposed albino mice by measuring the expression of 8-OHdG (photoproduct) and PCNA. **Method.** 25 mice were randomly divided into 2 groups: group A and group B. Group A was a control group consisting of 5 negative controls mice (without cocoa extract and without exposure to UVB), and 5 control mice (without cocoa extract but exposed to UVB). Group B consisted of 3 groups, which were given daily topical cocoa extract in various concentrations then exposed to UVB 450 mJ /cm<sup>2</sup> (3 times a week) for 12 weeks. All the animal were sacrificed 24 h after last exposure and samples were taken and processed for 8-OHdG and PCNA expressions. **Result.** For 8-OHdG expression, cocoa extract 100 ppm had shown protective effects which improving along with increasing dose of cocoa extract, but at 400 ppm protective effect has declined. It has been shown that cocoa extract 200 ppm gave the best protection against UVB exposure. For PCNA expression, extracts of 100 ppm and 200 ppm showed significant protective effect against UVB, but at 400 ppm the effect has declined. Similar to 8-OHdG expression, cocoa extract 200 ppm gave the best protective effect against UVB. **Conclusion.** The best concentration of cocoa extract to exhibit protective effect against UVB is 200 ppm.

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### 1. Introduction

Sunlight is a part of our daily life that are generally considered to have beneficial effects on health. However, there are some harmful effects of sunrays, both acute or chronic effects. Solar ultraviolet (UV) radiation is only 5% of all solar radiation that reaches the earth's surface. The spectrum of UV with a wavelength between 100 and 400 nm were divided into UVA1 (340-400 nm), UVA2 (320-340 nm), UVB (280-320 nm) and UVC (100-280 nm). Solar radiation that reaches the earth's surface is UVA 95-98%, and UVB 2-5%, while UVC entirely absorbed by the ozone layer in the stratosphere.

Exposure of human skin to UV radiation continuously is a major environmental factor that has a variety of effects such as erythema response and delayed pigmentation. Approximately 98-99% of erythema response occurs after exposure and reached a peak within 12-24 hours after exposure, and disappear within 3 days, that can be examined histologically known as sunburn cells or apoptotic cells. Chronic impact caused by repeated exposure to low energy levels are in the form of precancerous, skin cancer and premature aging. Epidemiological studies have showed an association between basal cell carcinoma and malignant melanoma with solar burns.

Cyclobutane Pyrimidine Dimer (CPD) are the most formed in the epidermis after UVB exposure and associated with direct DNA damage, whereas the 8-hydroxy-2-deoxyguanosine (8-OHdG)

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photoproduct more associated to DNA damage through the formation of oxidative stress. The photoproduct activates p53 expression. Induction of p53 initiate apoptotic cell formation, DNA repair, and increased of mitotic. Previous studies reported the formation of 8-OHdG in the epidermis of mice after repeated UVB exposure to 3.4 or 16.8 kJ / m<sup>2</sup> three times a week for 2 weeks, obtained an increase in 2.5 or 6.1-fold, respectively compared to unexposed controls.

Proliferating cell nuclear antigen (PCNA) is one of the main molecule responsible for the life or death decision of cells. PCNA was found in the basal layer of normal skin and in all layers of the epidermis on the malignancy. Immunohistochemical analysis revealed PCNA expression throughout the basal layer of untreated skin with diminished expression at 6 hours indicative of immediate UV damage and evidence by observable upregulation in pyrimidine dimer formation early on.

Then PCNA immunoreactivity increased progressively show excessive deviation epidermal migration patterns associated with chronic exposure. Reactivation of PCNA expression occurs with progressive improvement. PCNA migration to the upper layers of the epidermis indicates a potential increase proliferation and malignancy. PCNA gene is induced by p53 and p21 in the cell's life processes. When it does not play a role in DNA replication, PCNA (under the influence of p53) stop the cell to cell cycle and DNA damage repair, or when repairs cannot be done, the low functional levels of PCNA causes cells to undergo apoptosis.

One way to protect the skin from damage caused by UV radiation is using antioxidants. Some of the herbal remedies that have antioxidant activity has been studied as an anti-aging include green tea extract, grapeseed, soybean, berberine. Djawad et al 2008( unpublished) have showed protective effects of topical curcumin on the expression of photoproduct and hyperplasia in mouse epidermis.

In addition to the above herbal ingredients, herbal agents that have antioxidant effects are cocoa. Cocoa and cocoa products like chocolate contains polyphenols, especially flavonoids. Polyphenol (proanthocyanidins) is the end product of oligomers and polymers in the flavonoid biosynthetic pathway. The antioxidant activity in cocoa polyphenol content associated with it, especially the monomers catechin and epicatechin subunits. Final concentration of flavanols and antioxidant capacity of the final food products derived from cocoa depending on the type and origin of the cocoa beans, growing conditions, storage or post-harvest care and processing of products. Unfortunately, the production of chocolate conventionally can reduce the antioxidant capacity of cocoa beans. Fresh cocoa beans have higher antioxidant capacity compared to other food sources such as green tea, pomegranate, gojiberry, and blueberries. A study by Gasser et al using ex vivo human skin explants showed that topical application of cocoa polyphenols

increase glycosaminoglycan in the skin and increase collagen I, III, and IV. Waspodo et al, 2012 (unpublished) conducted a study experimental cocoa extract in mice that are exposed to UVB showed an increase in TGF- $\beta$  and decreased MMP1 which indicates improvement on the network.

Based on the problems and findings that cocoa is an antioxidant that protect the body from reactive oxygen species, and research on topical cocoa extract as protective of photoproduct expression has not been done, the research of protective effect of topical cocoa on skin from UVB exposure by assessing expression 8- OHdG (photoproduct) and PCNA.

### Material and Methods

This study was a true experiment research post test design with control group, which performed at the animal laboratory of Hasanuddin University. Immunohistochemistry was performed in the Laboratory Examination of Cytopathology 'Grand Medika' Makassar.

Subjects were 25 albino mice, Swiss albino mice species, females, aged 6-9 weeks, weighing 20-30 grams, healthy, and come from the same parent. Mice were maintained for at least 1 week under standard conditions: room temperature ( $28 \pm 2^{\circ}\text{C}$  temperature),  $50 \pm 10\%$  humidity and light cycle room with 12-hour on and 12-hour off. Mice were dead and sick during the study were excluded.

Mice were randomly divided into 2 groups : group A and group B. Group A was a control group consisting of 5 negative controls mice (without cocoa extract and without exposure to UVB), and 5 control mice that didn't expose to UVB but applied cocoa extract. Group B consisted of 3 groups, the first group applied 100 ppm topical cocoa extract, group II applied 200 ppm topical extract cocoa, group III applied 400 ppm topical extract cocoa. All mice were sheared midback, then the UVB control group and all of group B expose to UVB 450 mJ/cm<sup>2</sup> (3 times a week) for 12 weeks. The whole mice turned off after 24 hours from last intervention, then the skin on the back area taken of 2x2 cm in size for immunohistochemical examination, the 8-OHdG and PCNA.

Immunohistochemistry was performed using streptavidin-biotin-peroxidase metode and labeled with biotin streptavidin (Dako, Carpinteria, USA). The results of immunohistochemical examination will be grouped and total score 8-OHdG and PCNA interpretation was made, as follows (Irena Ranogajec et al, 2012): Score 0 (negative), a score of 1-3 (+1 = weak positive), a score of 4-6 (+2 = positive medium), and a score of 7-9 (+3 = strongly positive). On the positive result indicates the strength of the photoproduct expression dosage, whereas the negative results show photoproduct does not appear on the preparation.

## Result

The study was conducted on 25 mice which were divided into 5 groups. Each group consisted of a negative control group, the UVB control, cocoa 100 ppm, 200 ppm, and 400 ppm group, as showed in Table 1.

Natural materials were used in this study has been conducted with laboratory tests to determine the levels of cocoa polyphenols extracts as showed in Table 2.

**Table 1. Characteristics of distribution 8-OHdG and PCNA expression in all groups**

		Group A		Group B			P
		Negative Control	UVB Control	100 ppm	200 ppm	400 ppm	
8-OHdG	-	5	0	0	0	0	0,00
	+1	0	0	2	3	2	
	+2	0	3	2	2	3	
	+3	0	2	1	0	0	
PCNA	-	5	0	1	1	0	0,00
	+1	0	0	1	2	4	
	+2	0	4	3	2	0	
	+3	0	1	0	0	1	

Mann Whitney test

**Table 2. Polyphenol level on total cocoa extract**

Concentration	Cocoa Extract	
	Total Polyphenol Level	
100 ppm	7,42 µg	
200 ppm	14,48 µg	
400 ppm	29,68 µg	

**Table 3. Comparison of 8-OHdG expression between the control group with a group of daily cocoa extract application**

Group	8-OHdG expression				P
	-	+	++	+++	
Negative control	5	0	0	0	0,325
UVB control	0	0	3	2	
100 ppm	0	2	2	1	
200 ppm	0	3	2	0	
400 ppm	0	2	3	0	

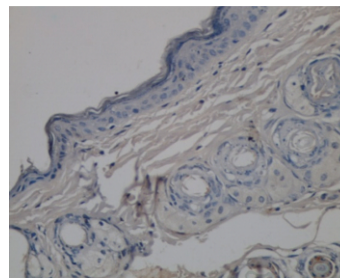
Kruskan Wallis test

**Table 4. Comparison of PCNA expression between the control group with a group of daily cocoa extract application**

Group	PCNA expression				P
	-	+	++	+++	
Negative control	5	0	0	0	0,665
UVB control	0	0	4	1	
100 ppm	1	1	3	0	
200 ppm	1	2	2	0	
400 ppm	0	4	0	1	

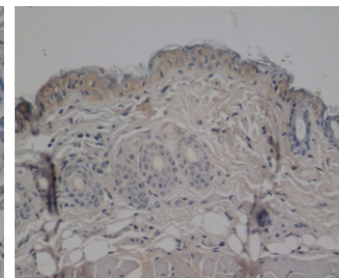
Kruskan Wallis test

## 8-OHdG Expression



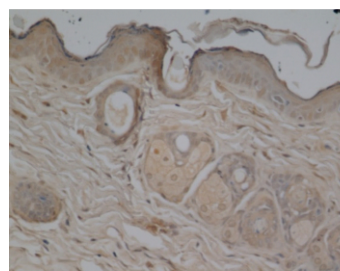
### Negative staining

There is no epithelial cells were stained.



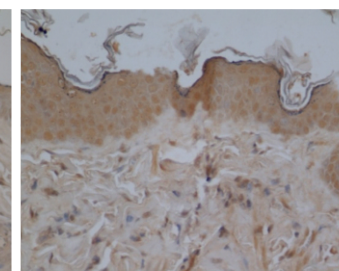
### Positive 1 / Low Expression

The brown stain on the nucleus and cytoplasm <25% of all epithelial cells in a single preparation



### Positive 2 / Moderate Expression

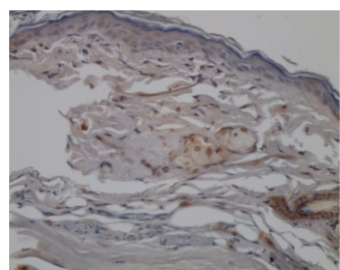
The brown stain on the nucleus and cytoplasm 25% - 50% of all epithelial cells in a single preparation



### Positive 3 / High Expression

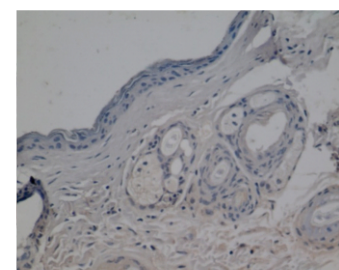
The brown stain on the nucleus and cytoplasm > 50% of all epithelial cells in a single preparation

## PCNA Expression



### Negative staining

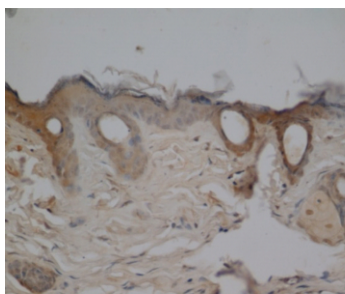
There is no epithelial cells were stained



### Positive 1 / Low Expression

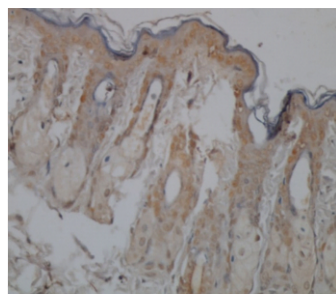
The brown stain on the nucleus and cytoplasm <25% of all epithelial cells in a single preparation





#### Positive 2/ Moderate Expression

The brown stain on the nucleus and cytoplasm 25%-50% of all epithelial cells in a single preparation



#### Positive 3/ High Expression

The brown stain on the nucleus and cytoplasm > 50% of all epithelial cells in a single preparation

Comparison of 8-OHdG expression between the control and daily application of cocoa extract group

On Table 3, comparison expression of 8-OHdG between control and daily application of extract cocoa can be seen that expression of 8-OHdG in the daily application of extract cocoa group with 100 ppm concentration there are 2 mice (+1), 2 mice (+2), 1 mice (+3), showed that the effect of the 100 ppm concentration is still approaching the control group were only UVB are exposed without any protection, it indicates this concentration only provide minimal protection effect.

Daily application of 200 ppm cocoa extract group, there are 3 mice (+1), 2 mice (+2) and there aren't in the (+3), indicate protection effect of 200 ppm cocoa extract due to the expression of 8-OHdG is not seen in the (+3), Table 3 seen shifted toward the control without any treatment. In daily application of 400 ppm cocoa extract group there are 2 mice (+1), 3 mice (+2), and does not exist in (+3), seen here even though cocoa extract concentrations higher than 200 ppm but in (+2) more (3 mice) compared to the (+1) (there are only 2 mice), it describe 8-OHdG expression or DNA damage more than 200 ppm concentration extract.

On Table 3, it is clearly seen that all the mice given the extract cocoa protection every day with various concentrations then exposed to UVB showed significant results, which shows that there is protection even though at 100 ppm there are mice in the (+3), but the concentration of 200 ppm and 400 ppm may no longer exist in mice (+3), on Table 3 leads to the negative control group (without any treatment) but concentration 200 ppm give the best protection.

On Table 3, the concentration 100 ppm have seen protective effects, with an increase in dose to 200 ppm, the effect is getting better, but at concentration 400 ppm, protective effect against UVB declined. Showed on Table 3 concentration 200 ppm give the best protection against UVB rays. Seen in the figures on the table, there are significant association between the change in concentration with the protective effect but the test was not statistically significant ( $p = 0.325$ ).

Comparison of PCNA expression between the control and daily extract cocoa application group

From Table 4 the figures for 100 ppm cocoa extract, starting to repair with 1 mice showed no damage on the skin, there is 1 mice with (+1) and 3 mice with (+2). The 200 ppm extracts cocoa group, gives better improvement with 1 mice showed no damage on the skin compare with mice given cocoa extract, 2 mice with (+1) (showing more than 100 ppm), 2 mice on the (+2). On the 400 ppm cocoa extract group, although this concentration is higher but showed no mice with negative results such as 100 ppm and 200 ppm concentration, 4 mice with (+1) and 1 mice with (+3). Base on various concentrations of cocoa extracts can be seen that the protection effect of cocoa extract which the concentration of 100 and 200 ppm showed 1 mice were negative result that mean no PCNA at both the concentrations. However, at higher concentrations, 400 ppm, showed strong expression of PCNA (+3) on 1 mice and weakly expression (+1) in 4 mice.

Table 4 describe clearly significant protection effect with application extract cocoa 100 ppm and 200 ppm which showed with mice in (-), with increasing concentrations to 200 ppm, lot of mice found on the (+) but with increasing dose to 400 ppm, the effect declined. Best protective effect of daily application cocoa extract showed on 200 ppm concentration, with significant number changes of mice at each concentration of cocoa extracts, although the test was not statistically significant ( $P = 0.665$ ).

### Discussion

The results showed 8-OHdG expression decrease along with application of cocoa extract topically. In the untreated control mice (without extract and UVB exposure), all mice showed no expression of 8-OHdG, while controls without cocoa extracts but expose to UVB showed high 8-OHdG expression. With the application 100 ppm extract cocoa, the expression of 8-OHdG decrease and decrease is more with application 200 ppm extract cocoa, this indicates that cocoa extracts provide a protective effect on the skin againsts UVB. 8-OHdG (8-hydroxy-2'-deoxyguanosine) is a product of oxidative DNA damage that induced by the action of hydroxyl radicals in DNA base deoxyguanosine and single chain DNA damage. Decrease in 8-OHdG showed a decrease in oxidative damage to DNA. DNA damage causes the initiation of carcinogenesis. Evidence suggests that the 8-OHdG reflect potential precancerous disease process. However, definite value of 8-OHdG as the prediction marker of cancer development is uncertain. Increased concentrations of cocoa extract statistically showed no association with 8-OHdG expression as showed by Kruskal Wallis test on various concentrations of cocoa extract was not significantly different but there is an increasing trend with increasing concentration effect except at 400 ppm concentration has decreased protective effect against UVB. Although research on topical application of cocoa extract containing polyphenols has not been a lot of high scientific data on the subject. The results of this study are supported by research conducted by Elmetts CA in 2001, said that the use of topical EGCG (catechin) before exposed to UV significantly decreased the formation of n of PCNA.

nitric oxide and hydrogen peroxyde production induced by UVB as well as leukocyte infiltration. It's believed that leukocyte infiltration is a major source of nitric oxide and oxidative stress. EGCG is reported have the ability to block the UVB-induced infiltration of leukocytes in mice and human skin and is able to inhibit the production of ROS induced by UVB through leukocyte infiltration. However, research by Gasser et al. using ex vivo human skin explants showed that topical application of polyphenols in chocolate improve skin glycosaminoglycan and increase collagen I, III and IV. Experimental research NN Wasposito et al using pure cocoa extract contain flavonoids that apply to mice exposed-UVB three times a week showed significant results in a 200 ppm concentration were associated with improved collagen (TGF-beta and MMP-I). In correlation with these results, daily application 200 ppm cocoa extract before exposed to UVB three times a week give the best effect to inhibit oxidative DNA damage (8-OHdG)

PCNA protein is one of the main molecule responsible for the life or death decision of cell. Almost all cellular processes involving DNA synthesis dependent and involves PCNA in the process. PCNA has a triple function in cell life and death. If not bind in DNA replication, PCNA (generally in control of P53) underwent arrest cycle and cell repair to DNA damage repair or if it not possible, the absence or low levels of functional PCNA can induce apoptosis. PCNA is a ring-like proteins that play a role in DNA replication and cells repair. PCNA expression can be used as a marker of cell proliferation because cells remain in a longer period of time during G1 / S phase in proliferation. From the results, the control without extract cocoa and exposure to UVB increased PCNA expression, the application of cocoa extracts with various concentrations decreased the expression of PCNA, 200 ppm concentration gives better results than 100 ppm, but with the increase in concentration to 400 ppm, PCNA expression decreased. Increased concentrations of cocoa extract statistically didn't effected the expression of PCNA as showed by Kruskal Wallis test using various concentrations of cocoa was not significantly different but there is a tendency increase with increasing concentration effect except at a concentration of 400 ppm, PCNA expression decrease.

On the 8-OHdG expression, increasing cocoa extract dose not followed by decreasing 8OHdG expression. With a concentration of 200 ppm daily topical application showed the most effective to decrease the expression of 8-OHdG, but increasing cocoa extract dose followed by decreasing protective effect against UVB exposure which were showed by the expression of 8-OHdG is not decreased. On PCNA expression, increasing doses of cocoa extracts didn't follow by decreasing expression of PCNA. Daily application 200 ppm cocoa extract showed maximum effect to reduce the expression of PCNA. Increasing doses of cocoa extract is not followed by an increasing the protective effect against UVB which with increasing doses of the extract increased the expressio

## Conclusion

Cocoa extract has a protective effect against ultraviolet radiation. To inhibit the expression of 8-OHdG, the best daily topical dose was 200 ppm. To inhibit the expression of PCNA, the best daily topical dose was 200 ppm. Increasing doses of cocoa extract has not extended its effectiveness in protecting against ultraviolet radiation. Therefore we suggest to do further research to determine the duration of the protective effect against ultraviolet from extract concentration that found and need a further investigation regarding other factors that may affect the expression of 8-OHdG and PCNA cocoa extract application.

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