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The Effectiveness of Topical 5-fluorouracil Treatment on Mouse Skin Squamous Cell Precancerous Lesions through Caspase-3 Expression

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Abstract

Skin cancer is a disease that develops in the epidermis of the skin and can be invasive, such as squamous cell carcinoma (SCC). Early detection of squamous cell precancerous can prevent these lesions from progressing to invasive SCC and increase the effectiveness of therapy. 5-fluorouracil (5-FU) is an antimetabolite compound as a pyrimidine DNA/RNA antagonist molecule that induces cell apoptosis. The main objective of this study was to evaluate the effectiveness of the topical 5-FU cream (Dharmais NCH) compared to imiquimod 5% on apoptosis through the expression of caspase-3 in precancerous squamous cells of mouse skin induced by 7,12-dimethylbenzen[a]-anthracene (DMBA)/croton oil treatment. This research assess three differences concentration of 5-FU include 1%, 2%, and 5% on 24 wild type mouse divided into 6 groups including positive control (with carcinogenesis but without treatment), negative control (without treatment; normal), carcinogenesis with treatment 5-FU cream (1%, 2%, and 5%) or 5% imiquimod cream. Two-stages carcinogenesis induced by DMBA and followed by croton oil. The expression of caspase-3 was analyzed using immunohistochemistry. Statistical analysis was performed by one-way ANOVA using SPSS version 23. The induction of two-stages of carcinogenesis (weeks 1 to 10) caused papilloma lesions on the skin of mouse. Furthermore, 5-FU treatment for 4 weeks (weeks 11 to 14) showed a decrease in the cumulative number of papillomas ($p<0.05$) and immunohistochemical analysis showed caspase-3 expression on 5-FU treatments (1%, 2%, and 5%) was not significantly different from the imiquimod treatment ($p>0.05$). The apoptotic effect of 5-FU treatment on precancerous skin squamous cell lesions in mouse was not significantly different from the standard treatment using imiquimod. This suggests that 5-FU treatment has potential as a future therapy in squamous cell precancerous skin lesions.

Keywords: *5-fluorouracil, caspase-3, squamous cell precancerous, skin, topical treatment.*

INTRODUCTION

The skin is the largest organ of the human body, accounting for 16% of total adult body weight (Knight, 2003). It performs many functions such as

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protection from many hazardous components from external environment and has an important role in thermoregulation (Igarashi, *et al.*, 2007). Human skin consists of three types of cells: squamous cells, basal cells, and melanocytes (Kolarsick, *et al.*, 2011). Every layer of the skin has risks for many diseases and nowadays skin cancer is a prominent threat for everyone. According to the International Agency for Research on Cancer (IARC) during 2008-2012, mortality and incidence of skin cancer worldwide relatively increased in both sexes around 8,202 and 14,068 per 100,000 individuals (WHO, 2012). Skin cancer is the most common among sun-sensitive populations, especially Caucasian and Indian populations (Chu, *et al.*, 2015). In Indonesia, the pathological-based cancer registry at Dharmais National Cancer Hospital (Dharmais NCH), Jakarta, for the period 1993-2012 showed that skin cancer has relatively increased ±10.29% every year (Division Cancer Registry, 2017)

Skin cancer is a multifactorial disease caused by heredity and several environmental factors (Netscher, *et al*., 2011). This disease develops in the epidermis and mainly causes three types including basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and melanoma. BCC is the most common type of skin cancer but relatively more treatable than other types (Singh, *et al*., 2014). Meanwhile, SCC is the second most common type of skin cancer, but it can become invasive if not treated in the early stages (precancerous lesion). In precancerous lesions, skin cells have DNA damage and which cause make them to proliferate uncontrollably (Singh, *et al*., 2014).

There are several routine treatments for SCC such as non-excision abrasion (electrodessication and curettage, cryotherapy, CO_2 laser, and photodynamic therapy), Mohs micrographic surgery, radiation, and topical treatment (imiquimod). However, those treatments have some limits including trauma, side effects, high cost, and the treatment must be performed by a doctor at a hospital (Eckes, *et al*., 2010). The effect of SCC therapy depends on the skin's condition because of cancer cell proliferation. Early detection of cancer increases recovery because the lesion has not become invasive and metastasized. One treatment that has been developed in Dharmais NCH is the use of 5-Fluorouracil (5-FU) topical treatment (concentration 1%, 2%, and 5%) for squamous cell precancerous lesions.

5-FU is an antimetabolite compound that is a pyrimidine antagonist for DNA and RNA synthesis (Ceilley, 2012). This compound enters the cell in the same way as uracil where it is changed to be an active metabolite such as fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridin triphosphate (FUTP) (Zhang, *et al*., 2008). The 5-FU induces intracellular reactive oxygen species (ROS) and activation of the ERK/AKT pathway (Fan, *et al*., 2013), which causes activation of p53 and dysfunction of mitochondria to release cytochrome c leading caspase-3 activation and the intrinsic apoptosis pathway (Chinembiri, *et al*., 2015). This research was designed to evaluate the effectivity of the 5-FU cream from Dharmais NCH for squamous cell precancerous lesion treatment through caspase-3 expression compared with imiquimod 5% cream, which is a standard treatment.

MATERIALS AND METHODS

Animals

Twenty four female Swiss Webster mouse 6 weeks of age divided into six groups (four mouses/group) based on Frederer formula (Frederer, 1967); 1) Positive control carcinogenesis without treatment, 2) Negative control without treatment (normal), 3) Carcinogenesis with 5-FU treatment 1%, 4) Carcinogenesis with 5-FU treatment 2%, 5) Carcinogenesis with 5-FU treatment 5%, and 6) Carcinogenesis with imiquimod treatment 5%. The mouse were obtained from the animal facility of the Animal Laboratory, Agency of Health Research and Development, The Indonesian Health

Ministry. The animals weighed between 20-30 g and were pathogen-free. The mouse were kept in a room with ventilation (12/12h; light/dark cycle), controlled temperature (19.3-23°C), relative humidity (44-74%), and given water and a balanced diet *ad libitum*. This study has been approved by Ethics Committee, Faculty of Medicine, Universitas Indonesia and Cipto Mangunkusumo Hospital (approval number: 1159/UN2.F1/ETIK/2017).

Carcinogenesis Model

The carcinogen 7,12-dimethylbenzen[a]-anthracene (DMBA) (Sigma-Aldrich, St. Louis, Missouri, USA) was used in at concentration of 500 nmol/100 µL acetone. Each animal in the carcinogenesis group treated with DMBA twice a week topically at the back (Sati, *et al*., 2016). After two weeks the animals were topically treated with croton oil (CO) (Sigma-Aldrich) in concentration 1%/100 µL acetone three times a week (Qiblawi & Dhanarasu, 2017) for 10 weeks. For the treatment groups, these mouse were treated using 5-FU cream (1%, 2%, and 5%) one daily and imiquimod 5% cream two times a week topically for 4 weeks while the positive control groups continue being treated with croton oil until week-14 (Figure 1). All the animals were euthanized by cervical dislocation at week-14.

Figure 1. Step of the treatments.

Histopathological Study

All mouse tumors tissues and normal skin tissues were fixed in formalin (10%), embedded in paraffin, and sections (5 µm) using a rotary microtome and stained with hematoxylin and eosin (HE) (Arora, *et al*., 2011). Slides are evaluated under the light microscope and captured using Aperio AT Turbo Slide scanner (Leica Biosystem).

Immunohistochemistry Staining

Paraffin blocks were sliced into a 4 µm-thickness with a microtome and the tissue slices were fixed on positive charge slides (Aurona Technologies) overnight. For positive control were used tonsil tissue. The slides were heated to 60°C for 1 h and were deparaffinized using xylene (3×5 minutes) and serial alcohol (3×5 minutes). Antigen retrieval was performed using Tris-EDTA pH 9.0 by boiling and cooling down (×3) in a microwave oven (Frat, *et al*., 2012). The slides then were washed with phosphate buffer saline twice for 5 minutes each. Endogenous peroxidase blocking was done with Peroxide Block (UltraTek Complete HRP Anti-Polyvalent (DAB) staining system, Scytex Laboratories Inc.) for 10 minutes at room temperature (RT), then Super Block for 20 minutes at RT. Subequently, the slides were treated with rabbit anti-human caspase-3 polyclonal antibody (1:500) (Ab gtx73090, GeneTex,) and incubate in a moist chamber (RT) for 30 minutes. After that, the slides continued to incubate on UltraTek Complete HRP Anti-Polyvalent for 10 minutes at RT and UltraTek HRP for 10 minutes at RT. Finally, DAB chromogen/substrate was applied for 3 minutes at RT and then counterstained. The slides were evaluated under the light microscope and capture using Aperio AT Turbo Slide scanner. Scoring was done by pathologist using ImageG 1.50i software (0; negative, +; low positive, ++; positive, +++; high positive). The scoring number automatically appears after the pathologist run plugins IHC profiler then select cytoplasmic stained image and H-DAB.

Table 1. Body weight of mouse during the study.

Treatment	BW (gr)*						
	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14
Normal (negative control)	24.75±2.06	27±2.16	27.75±2.22	28.25±1.50	29.75±2.63	29±2.45	28.5±2.38
DMBA/CO (positive control)	27±0.82	27±1.83	27.25±3.10	27.75±2.06	29±1.83	28.75±2.22	28.5±2.38
DMBA/CO+5-FU 1%	24±2.16	24.5±2.38	24.75±2.50	25.25±2.50	26.25±2.36	26.25±1.71	24.75±3.30
DMBA/CO+5-FU 2%	26.25±2.06	26.5±2.38	28±3.56	27.25±2.50	29.75±3.30	29.75±3.30	29±4.69
DMBA/CO+5-FU 5%	26.75±1.26	26.75±1.50	26.25±3.10	27.25±2.99	29.25±1.89	29.25±2.06	28.75±1.50
DMBA/CO+Imiquimod 5%	26.5±1.00	26.5±1.00	28±1.41	28.25±1.71	30±1.63	29.25±2.50	30±1.83

*Body weight (BW) are expressed as mean ±SD (n=4)
Statistical analysis was determined by ANOVA and indicate no significant difference among the groups (p>0.05).

Statistical Analysis

Statistical analysis was performed using SPSS version 23. Two-way ANOVA was used to assess the data followed by Bonferroni test. The significance level was set at p<0.05 for all experiment.

RESULTS

In this research, the mouse body weight was calculated during the research process and all treatments had no effect on it (Table 1). It was shown by ANOVA analysis with the result that there was no significant difference among the groups (p>0.05).

Furthermore, based on ANOVA analysis there were significantly different number and volume of papillomas among the groups (p=0.001). It showed that during treatment that there was an improvement in skin epithelial cells as indicated by a decrease in the number and volume of papil (Table 2). Bonferroni analysis showed that there was significantly different between positive control group (DMBA/CO) compared to 5-FU 5% and imiquimod 5% (p=0.003). This indicates that 5-FU 5% and imiquimod 5% treatments resulted

Table 2. Cumulative number and volume of papilloma in mouse before and after treatment.

Treatments	Before treatment		After treatment	
	Number of papil*	Volume of papil (mm^3)*	Number of papil*	Volume of papil (mm^3)*
Normal (negative control)	0.00±0.00	0.00±0.00[a]	0.00±0.00	0.00±0.00[a]
DMBA + Croton oil (positive control)	3.50±1.29	0.27±0.29	4.00±1.41	0.26±0.21
5-FU 1% treatment	4.00±2.16	5.30±7.21[b]	3.25±2.22	1.04±2.04[b]
5-FU 2% treatment	2.75±0.96	1.67±2.22	2.75±0.96	0.35±0.62
5-FU 5% treatment	2.25±0.96	1.32±1.65[a]	2.00±0.82	1.05±0.54[a]
Imiquimod 5% treatment	2.50±1.00	2.30±3.47[ab]	2.00±0.82	0.40±0.31[ab]

*Number and volume of papil (mm^3) are expressed as mean ±SD (n=4)
[a,b] showed significantly different (p<0.05) using ANOVA followed by Bonferroni analysis

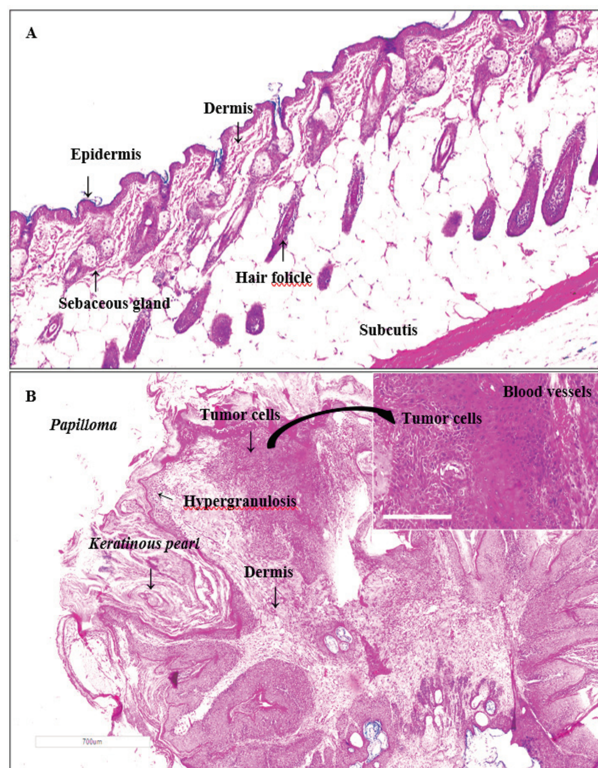

Figure 2. Normal skin structure taken from negative control group. (A) Papilloma skin structure taken from the positive control (DMBA/CO) group. (B) Were stained with hematoxylin and eosin. Scale bar=40x and 100x.

in skin improvement with reduced papilloma volume compared to the untreated group. Furthermore, there was a significant difference between 5-FU 1% compared to imiquimod 5% treatment (p=0.000). It means that 5-FU 1% treatment gave a different therapeutic effect with imiquimod 5% compared to the 5-FU 2% and 5% treatment.

HE staining (Figure 2) showed that there was a skin structure change after two-stage carcinogenesis. It showed that the normal skin structure containing a clear epidermis layer, dermis, and subcutis (Figure 2A). Normal skin epidermis consist of stratified squamous epithelium which contain of basal cell layer, prickle cell layer, granular cell layer, and keratin layer. However, after two-stage carcinogenesis (Figure 2B) showed that the squamous epithelial layer has changed and cannot be clearly distinguished. HE staining of papillomas or precancerous squamous cell lesions shows dysplasia, stromal damage, hyperkeratosis, acanthosis, and keratinous pearl.

Caspase-3 expression was observed as brown staining in the cytoplasmic cell and this was considered to be positive (Figure 3). For caspase-3 immunohistochemistry, tonsil was used for the positive control (Figure 3A). Positive controls are specimens containing the target molecule in its known location. Negative control are specimens from tissue without target molecule (Figure 3B). Figure 3C was a squamous cell precancerous specimen without treatment administration while Figure 3D was a squamous cell precancerous specimen after treatment with 5-FU. Figures 3A, 3C, and 3D show all positive specimens containing caspase-3 molecules which are marked with brown color in the cytoplasmic area.

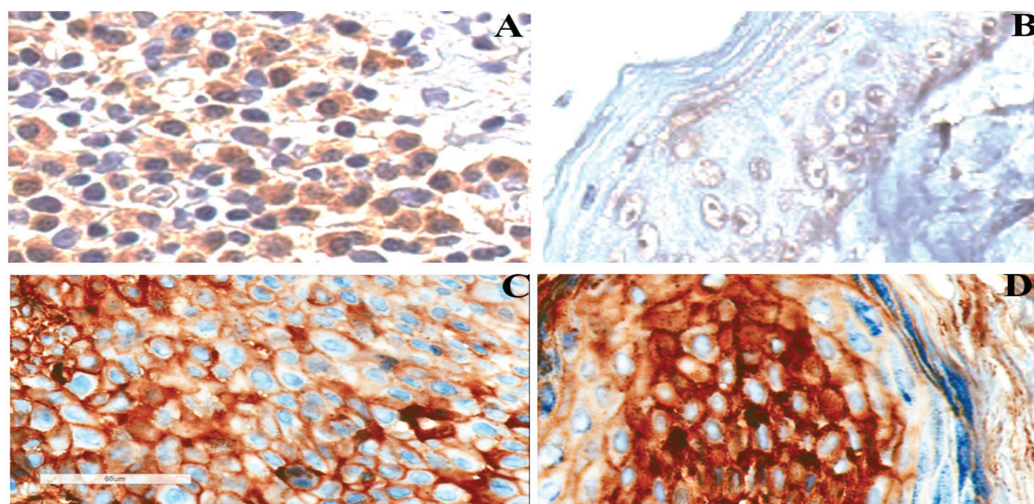

Figure 3. The caspase-3 positive cells were shown in brown color in the cytoplasmic area. (A) Positive expression in the positive control (tonsil tissue). (B) Negative expression in the negative control. (C) Positive expression in two-stage carcinogenesis skin without 5-FU treatment. (D) Positive expression with 5 FU treatment. Scale bar=400x.

Table 3 shows that the lowest caspase-3 expression is in normal control group (1.04±0.01) with a low score positive (+), for therapy groups the highest caspase-3 expression is in the imiquimod 5% group (2.90±0.26) (+++), while the lowest in 5-FU 1% (2.25±0.69) (++). Statistical analysis showed a significant difference among the groups using ANOVA analysis ($p<0.05$). Furthermore, Bonferroni's analysis showed only the normal group compared to all therapy groups were significantly different ($p<0.05$) while there was no significant difference between treatment groups ($p>0.05$). This result showed that the use of topical 5-FU 1%, 2%, and 5% concentration (made by Dharmais NCH) provides results that were not different from standard topical imiquimod therapy in squamous cell precancerous lesions. Therefore, the use of topical 5-FU can be used as a clinical trial material in the future.

Table 3. Optical density score for caspase-3 expression for all groups.

Treatment	Optical density Score
Normal	1.04±0.01*
DMBA/CO (untreated control)	2.59±0.26
DMBA/CO+5-FU 1%	2.25±0.69
DMBA/CO+5-FU 2%	2.79±0.42
DMBA/CO+5-FU 5%	2.47±0.42
DMBA/CO+Imiquimod 5%	2.90±0.26

* showed significantly different ($p<0.05$) using ANOVA followed by Bonferroni analysis.

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DISCUSSION

Skin cancer, especially squamous cell carcinoma, can be caused by the carcinogenesis process. Two-stage carcinogenesis was used in this study by inducing normal mouse skin into squamous cell precancerous lesions. The carcinogen DMBA consist of polycyclic hydrocarbon that causes a DNA adduct as an initiation stage of carcinogenesis in the skin. DMBA become active in the liver by modulating phase-1 detoxification enzyme cytochrome P450 and converts to 3,4-diol-1,2-epoxide which covalently bind to DNA (DNA adduct) (Sharma & Goyal, 2015). Moreover, this activation causes a mutation in the Hras1 gene (67%), Kras gene (19%), a transversion of A→T at codon-61 (Abel, *et al*., 2011), and DNA methylation in Nr4a3 exon 3 (Nassar, *et al*., 2015).

Following the initiation stage, the cells are promoted clonally by croton oil (promotion stage). Tumor promotion leads to sustained epidermal hyperplasia evidenced by an increase in the number of nucleated cells and increase the thickness of epidermis. This initiated cell growth is independent over neighboring cells and loss of cell population. The result of this promotion stage is development of skin papilloma or squamous cell precancerous lesion (Subramanian, *et al*., 2014). All mouse topically treated with DMBA/CO in this research developed papilloma within 5/6 weeks. During the two-stage skin carcinogenesis and followed by treatment, the mouse body weight is not affected (Table 1). It caused by nutrition and environmental conditions are always maintained so that the mouse do not lose weight due to stress or treatment during the research.

Skin papilloma consists of a stromal core surrounded by hyperplastic epidermis and squamous epithelial layer cannot be clearly distinguished (Figure 2B) compared to normal skin condition (Figure 2A). This promotion stage increased epidermal thickness, proliferation of epidermal keratinocytes, dan increased DNA mutated synthesis. Moreover, the epidermal keratocytes are immature, thereby contributing to parakeratosis alternating with hyperkeratosis (Rowert-Huber, *et al*., 2007). Continous carcinogen exposure develop cells proliferate or divide uncontrollably and form precancerous lesions (papilloma) (Table 2). In addition, apoptosis control is an important factor to cellular homeostasis regulation. When cells receive an apoptosis signal, the apoptosis gene will be activated and trigger the apoptosis pathway (Gu, *et al*., 2015).

Caspase-3 is one protein from the cysteine protease class that acts as an executor caspase and is often used as an apoptosis marker (Alexandrakis, *et al*., 2004). Caspase-3 can be activated by extrinsic and intrinsic mechanisms. The expression of caspase-3 protein in some studies will increase in the tissue indicating tumor growth or hyperplasia as in breast cancer, gastric adenocarcinoma, and stomach cancer, while in non-hyperplasia tissue will decrease (Wang, *et al.*, 2017). In this research, caspase-3 analyzed using immunohistochemistry method. Caspase-3 expression was observed as brown staining in the cytoplasmic cell, and this was considered to be positive. Kuranaga explained that increased caspase-3 expression in benign tumors such as precancerous lesions is due to the cell's attempts to maintain homeostatic conditions by activating caspase-3 and trigger the apoptosis mechanism (Kuranaga, 2012). The result showed all the specimen from papilloma lesion were positive, but different intensities depend on the treatment given (Figure 3 and Table 3). 5-FU treatment is known to inhibit the action of thymidylate synthase by binding to DNA or RNA causing an interruption of nucleotide metabolism (Zhang, *et al.*, 2008). An apoptosis induction by a 5-FU anticancer compound encourages caspase-3 expression (Coutinho-Camillo, *et al.*, 2017).

Caspase-3 expression on 5-FU treatment is known to have intrinsic apoptosis pathways through potential membrane damage to cell mitochondria. This damage is stimulated by Bax

translocation from the cytosol into the mitochondria and releasing cytochrome-c. Cytochrome-c release into the cytosol binds to Apaf-1 and activates procaspase-9 in the apoptosome. Caspase-9 activation causes activation of the caspase executor, in this case caspase-3 which will fragment the target protein including poly (ADP-ribose) polymerase resulting in cell death (Coutinho-Camillo, *et al.*, 2017). Caspase-3 can activate many substrates in the nucleus such as lamin A, actin, and α-fodrin which further causes the cell to contract and the cell membrane becomes irregular. Caspase-3 also activates CAD which causes DNA fragmentation, core degradation including cytoskeleton protein, and cell disintegration into apoptotic bodies (Rastogi, *et al.*, 2009; Elmore, 2007). However, topical imiquimod treatment (standard therapy) has been shown to be effective and induces apoptosis by activating caspase-3 in skin cancer (melanoma, BCC, and SCC) and precancerous (actinic keratosis) lesions (El-Khattouti, *et al.*, 2016; Walter, *et al.*, 2013). It acts as an immunomodulator by inducing toll-like receptor 7 and or 8, which induces helper T cells and dendritic cells thus activating cytotoxic T cells resulting in tumor lysis (Love, *et al.*, 2015; Sohn, *et al.*, 2014).

Positive caspase-3 expression in this research is an indication that the apoptosis mechanisms are still working for each treatment. The expression of caspase-3 protein signifies that in the tissue there has been homeostasis between proliferating cells and apoptosis. Activation of caspase-3 also has a role to stimulate non-apoptosis cells in the vicinity to proliferate as tissue regeneration and speed up the wound healing process (Pollard, *et al.*, 2008).

CONCLUSION

5-FU treatment for 4 weeks in pre-cancerous skin lesions showed a decrease in the cumulative number and volume of papillomas. However, the apoptotic effect of 5-FU treatment on precancerous skin squamous cell lesions for 4 weeks in mouse was not significantly different from standard treatment using imiquimod. Thus, this suggests that 5-FU treatment has potential as a future therapy in squamous cell precancerous skin lesions.

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