



**UNIVERSITI PUTRA MALAYSIA**

***THE EFFECTS OF C5aR ANTAGONISM ON HISTOPATHOLOGICAL  
AND BLOOD PARAMETERS CHANGES FOLLOWING CHLORHEXIDINE-  
INDUCED CONTACT DERMATITIS IN MICE***

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HISTOPATHOLOGICAL AND BLOOD PARAMETERS  
CHANGES FOLLOWING CHLORHEXIDINE-INDUCED  
CONTACT DERMATITIS IN MICE**

**SIONG JING JING**

A project paper submitted to the  
Faculty of Veterinary Medicine, Universiti Putra Malaysia

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It is hereby certified that we have read this project paper entitled “The Effects of C5aR Antagonism on Histopathological and Blood Parameters Changes Following Chlorhexidine-Induced Contact Dermatitis in Mice” by Siong Jing Jing and in our opinions it is satisfactory in term of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4901-Project.

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DEDICATION

*Every challenging work needs self-effort as well as guidance of elders especially those who were very close to our hearts.*

*My humble effort I dedicate to the Al-mighty*

*God*

*All the support and care from my loving*

*Father and Mother*

*Along with all responsible and respected*

*Lecturers*

*As well as all guidance and assistance from*

*Laboratory staffs and friends*

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**LIST OF ABBREVIATIONS**

|       |                                             |
|-------|---------------------------------------------|
| CD    | contact dermatitis                          |
| CICD  | chlorhexidine-induced contact dermatitis    |
| ACD   | allergic contact dermatitis                 |
| ICD   | irritant contact dermatitis                 |
| C5L2  | C5a receptor-like 2                         |
| C5aR  | C5a receptors                               |
| IACUC | Institutional Animal Care and Use Committee |
| UPM   | Universiti Putra Malaysia                   |
| H&E   | haematoxylin and eosin                      |
| WBC   | white blood cell                            |

## **ABSTRAK**

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar  
untuk memenuhi sebahagian daripada keperluan kursus VPD4901-Projek

### **KESAN ANTAGONIS C5aR PADA PARAMETER HISTOPATOLOGI DAN PERUBAHAN DARAH BERIKUTAN KONTAK CHLORHEXIDINE DENGAN KULIT MENYEBABKAN KERADANGAN KULIT DALAM TIKUS**

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2015

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Penyelia bersama: Prof. Noordin Mohamed Mustapha

Sepanjang beberapa dekad, chlorhexidine merupakan salah satu bahan disinfektan yang kerap digunakan dalam bidang perubatan sama ada untuk manusia atau haiwan. Namun, chlorhexidine telah didapati bahawa bahan ini akan mengakibatkan reaksi memudaratkan seperti hipersensitiviti dalam manusia, tetapi sama ada bahan ini

boleh menghasilkan tindak balas yang sama pada haiwan masih tidak jelas. Dalam kajian ini, kami akan menyiasat tentang kesan antagonis C5aR pada parameter histopatologi dan perubahan darah berikutan kontak chlorhexidine dengan kulit menyebabkan keradangan kulit dalam tikus. Tikus secara rawaknya dibahagikan kepada empat kumpulan untuk menyebabkan chlorhexidine kontak dermatitis atas kulit dan rawatan Dexamethasone, Histamil, komponen pelengkap C5a reseptor antagonis PMX205 dan air masin natrium klorida diberikan kepada kumpulan masing-masing. Sampel-sampel darah diambil untuk menghasilkan keputusan hematologi manakala sampel kulit diambil untuk penilaian keputusan histopatologi. Kemungkinan hasil serum IgE dalam darah melalui tindak balas alahan pula dikesan dengan teknik ELISA. Keputusan yang diperolehi daripada kajian ini menunjukkan bahawa rawatan chlorhexidine kontak dermatitis dengan C5aR antagonis telah berjaya mengurangkan keterukan luka yang serupa dengan anti-histamine dan rawatan corticosteroid berdasarkan pemarkahan lesi kulit secara kasar dan penilaian histopatologi. Keputusan hematologi untuk darah tidak menunjukkan apa-apa perubahan ketara berbanding dengan normal. Sebagai ringkasan, C5aR percanggahan menyediakan rawatan alternatif kepada chlorhexidine kontak dermatitis.

**Kata Kunci:** *chlorhexidine, chlorhexidine kontak dermatitis, C5aR antagonis*

## **ABSTRACT**

An abstract of the project paper presented to Faculty of Veterinary Medicine in  
partial fulfilment of the course of VPD 4901-Project

### **THE EFFECTS OF C5aR ANTAGONISM ON HISTOPATHOLOGICAL AND BLOOD PARAMETERS CHANGES FOLLOWING CHLORHEXIDINE-INDUCED CONTACT DERMATITIS IN MICE**

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2015

Supervisor: Dr. Mohd Hezmee bin Mohd Noor

Co-supervisor: Prof. Noordin Mohamed Mustapha

For decades, chlorhexidine has been used in the medical field for its disinfectant properties. Yet, chlorhexidine is found to illicit hypersensitivity reactions in human, but whether it can produce the same reaction in animals remain unclear. In this study, we investigate the effects of C5aR antagonism on histopathological and blood parameters changes following chlorhexidine-induced contact dermatitis in mice.

Mice were randomly divided into four groups for chlorhexidine induced contact dermatitis and given treatments of Dexamethasone, Histamil, complement component C5a receptor antagonist PMX205 and saline. Blood samples were taken for haematology while skin samples were taken for histopathology analysis. The viability of serum IgE for allergy reaction is detected by ELISA technique. Results obtained from the study indicate that treatment of chlorhexidine-induced contact dermatitis by C5aR antagonist has managed to reduce the severity of the lesions similar to anti-histamine and corticosteroid treatment based on the gross skin lesion scoring and histopathological evaluation. The blood parameters did not showed any significant changes compared to normal. As a summary, C5aR antagonism provides an alternative towards the treatment of chlorhexidine-induced contact dermatitis.

**Key Words:** *chlorhexidine, chlorhexidine-induced contact dermatitis, C5aR antagonist*

## 1.0 INTRODUCTION

For decades, chlorhexidine has been highly utilized in the medical field for its broad-spectrum efficacy, microbicidal properties and low costs. Although exposure to chlorhexidine is very common in the health-care settings, sporadic cases of contact dermatitis (CD) occur regularly and are well documented, especially in the health-care workers in the medical and surgical environment. Chlorhexidine is found to illicit hypersensitivity reactions in human, but whether it can produce the same reaction in animals remain unclear. Studies have shown that IgE-mediated chlorhexidine allergy may lead to type I allergy reactions, extending from mild symptoms to anaphylaxis in human (Garvey et al., 2007). Currently topical corticosteroid is one of the most favoured treatments for cases of CD (Cohen & Heidary, 2004; Krautheim, Jermann, & Bircher, 2004). However, prolonged use of corticosteroid may not be warranted for chronic cases due to several complications such as polydipsia, polyuria, liver problems, muscle atrophy and susceptibility to skin and bladder infections (Koch, Torres, & Plumb, 2012). Therefore it is necessary to search for different methods to reduce the treatment of this disease with minimal side effects. A complement system is responsible to assist in the antibody action toward destroying the pathogen and eliminate the antigen-antibody complexes. Out of the complement components, C5a is important as an inflammatory mediator in most of the diseases. Hence, as an inflammation mediator, the idea of using the C5a antagonism to reduce and prevent the inflammation process can be manipulated in treating contact dermatitis (CD).

## **2.0 LITERATURE REVIEW**

### **2.1 Chlorhexidine**

Chlorhexidine is a commonly used antiseptic agent in health-care settings (Toholka & Nixon, 2013). As a cationic diacetate or digluconate salt, chlorhexidine has bactericidal effects against gram-positive and gram-negative bacteria. It also has a bacteriostatic action in low concentrations, and is active against some bacterial spores and viruses. In addition, chlorhexidine also has an antifungal activity, which can reduce the amount of, for example, skin colonizing yeasts, including *Malassezia* and various *Candida* species. Chlorhexidine is commonly used for skin and mucosal disinfection in medicine and dentistry. Oral hygiene products, topical therapies and cosmetics include chlorhexidine salts as biocides or preservatives (Autegarden, Pecquet, Huet, Bayrou, & Leynadier, 1999).

### **2.2 Chlorhexidine-induced contact dermatitis (CICD)**

Contact dermatitis represents an inflammatory skin reaction towards external agents, such as an irritant or an allergen. Allergic contact dermatitis (ACD) involves a delayed type hypersensitivity reaction and need a prior sensitization to elicit a response, whereas irritant contact dermatitis (ICD) does not primarily require a prerequisite T cell response to initiate a reaction (Cohen & Heidary, 2004). Although exposure to chlorhexidine is very common in medical and veterinary practices, it is barely known by the public for causing ACD in human (Goon, White, Rycroft, & McFadden, 2004; Toholka & Nixon, 2013). Since its introduction in 1954 as an antiseptic and disinfectant, multivarious hypersensitivity reactions to this agent have

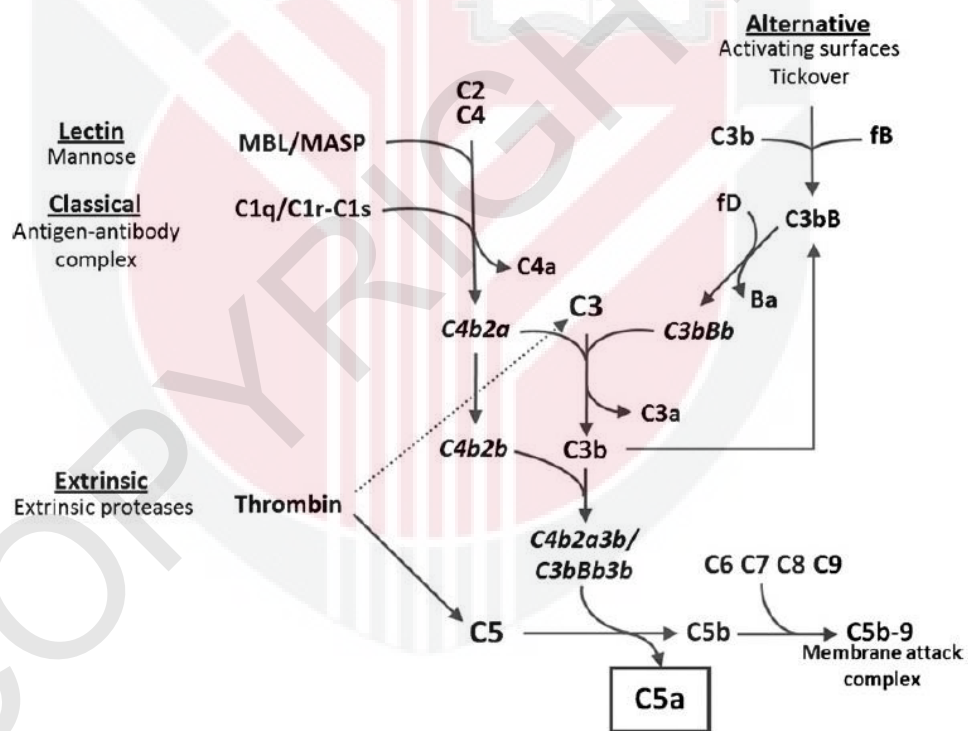
been reported, including delayed hypersensitivity reactions such as CD (Lasthein Andersen & Brandrup, 1985). However, such incident is still not clearly investigated in animals as there is no source of articles or case reports could be found to prove it.

### 2.3 Complement C5a

C5a is a potent anaphylatoxin and chemoattractant that is generated from C5 as a part of both the classic and alternative pathways of the complement activation (Bhatia et al., 2001). It was first described as a classical anaphylatoxin, capable of stimulating the secretion of histamine from mast cells (Friedberger, 1910), and later identified as a potent neutrophil (Becker, 1972; Snyderman, Phillips, & Mergenhagen, 1970) and macrophage (Snyderman, Pike, McCarley, & Lang, 1975) chemoattractant. C5a was first described as a cleavage product of complement factor 5 (C5) with chemotactic and anaphylatoxic properties (Shin, Snyderman, Friedman, Mellors, & Mayer, 1968). Further characterisation revealed that C5a is an essential part of the innate immune response and evidence now suggests that it may also play a role in adaptive immunity (Kohl, 2006).

The complement system is composed of over 30 proteins, activated in response to tissue injury, invading pathogens or other foreign surfaces (Manthey, Woodruff, Taylor, & Monk, 2009). The complement cascade is activated via three pathways (**Figure 1**): the classical, the lectin, and the alternative (Ricklin & Lambris, 2007). The classical activation pathway is a response to the formation of immune complexes of complement fixing IgG and IgM antibodies (Monk, Scola, Madala, & Fairlie, 2007; Vollmers & Brandlein, 2006). The second lectin activation pathway is

initiated by lectins, which recognize the glucose structures that decorate the surfaces of infectious organisms (Monk et al., 2007). The third alternative pathway relies on the continuous degradation of component C3 that occurs on pathogen and host cell surfaces (Monk et al., 2007; Thurman & Holers, 2006). All three pathways result in the formation of C3 convertases which cleave C3 to form C3a and C3b. Instead of just responsible for opsonising pathogen surfaces for recognition and clearance, C3b also forms part of the C5 convertases (C4b2aC3b or C3bBbC3b) that cleaves C5 to yield C5a and C5b.



**Figure 1:** The complement cascade. C5a is formed via activation of the complement cascade. Activation via the classical pathway, alternative pathway and the lectin pathway results in the formation of the C5 convertase (C4b2a3b or C3bBb3b) which cleaves C5 to form C5a and C5b. Activation via the extrinsic pathway results in the direct cleavage of C3 and C5 by extrinsic proteases such as thrombin.

The complement cascade is closely regulated by a series of soluble and membrane-bound regulatory proteins which prevents complement activation products from targeting host tissues (Ricklin & Lambris, 2007). However, this control can be bypassed by extrinsic pathways that involve direct cleavage of C3 and C5 by proteases such as thrombin (Amara et al., 2008). Furthermore, activated neutrophils and alveolar macrophages can generate C5a from C5 with secreted serine proteases (Amara et al., 2008).

Upon cleavage from C5, C5a is quickly metabolised by plasma and cell surface (Bokisch & Muller-Eberhard, 1970), then quickly cleared from the body circulation within 2-3 minutes, by binding to the classical C5a receptor, CD88, which is available on the leukocytes and other cells (Oppermann & Gotze, 1994). However, the second receptor, C5a receptor-like 2 (C5L2), which is more effective at the removal of component fragment, by rapidly internalising C5a, where it is retained and, in some cell types, degraded (Scola, Johswich, Morgan, Klos, & Monk, 2009). In contrast, cells expressing CD88 internalise C5a but release a higher proportion in an undegraded, possibly still active form. Plasma C5a may also be cleared by the liver (Chenoweth & Goodman, 1983).

#### **2.4 C5a antagonist for anti-inflammatory therapy**

Complement activation products, including C5a, are thought to play a role in the pathogenesis of many inflammatory and immunological diseases (Guo & Ward, 2005; Woodruff et al., 2006). Elevated levels of C5a have been found in the serum of patients with inflammatory disorders. Overexpression or under-regulation of C5a is implicated in human or experimental models of inflammatory conditions, such as

sepsis (Huber-Lang et al., 2002; Ward, 2004), adult respiratory distress syndrome (Hammerschmidt, Weaver, Hudson, Craddock, & Jacob, 1980; Robbins, Russ, Rasmussen, & Clayton, 1987), rheumatoid arthritis (Grant et al., 2002; Linton & Morgan, 1999; Woodruff et al., 2002), glomerulonephritis (Kondo et al., 2001; Welch, 2002), ischemia-reperfusion injury (Arumugam et al., 2003; Arumugam, Shiels, Woodruff, Granger, & Taylor, 2004; Martin, Chenoweth, Engler, Roth, & Longhurst, 1988; Proctor et al., 2004), allergy and asthma (Abe et al., 2001; Baelder et al., 2005; Gerard & Gerard, 2002; Hawlisch, Wills-Karp, Karp, & Kohl, 2004; Lambrecht, 2006), meningitis (Bhatia, 2002), neurodegeneration and macular degeneration (Kijlstra, La Heij, & Hendrikse, 2005; van Beek, Elward, & Gasque, 2003), following major trauma (Sewell et al., 2004), burns (Piccolo et al., 1999) and infection.

The excessive or uncontrolled production of C5a that occurs in these inflammatory diseases has suggested that C5a promotes and perpetuates inflammatory reactions (Guo & Ward, 2005). Thus, there is considerable interest in the development of therapeutics to block these pro-inflammatory effects (Manthey et al., 2009). Bioavailable antagonists could conceivably have potent anti-inflammatory properties in many diseases (Monk et al., 2007). Studies have shown that genetically altered knockout mice that lack C5a receptors (C5aR) are more susceptible to bacterial infection but protected against immune complex-mediated lung injury (Bhatia et al., 1998; Hopken, Lu, Gerard, & Gerard, 1996). It also demonstrated the significant of C5aR antagonist in reducing the lesion sizes, clinical signs severity,

associated apoptosis, and neutrophil infiltration in rodent models with neurodegenerations (Manthey et al., 2009; Woodruff et al., 2006).

A numbers of inhibitor of C5a have been developed due to the advantage of specific inhibition of C5a activity for not interfering with the other, protective actions, of complement (Ricklin & Lambris, 2007; Woodruff et al., 2006). Conceivably the most promising and therapeutic candidate and undoubtedly the most widely used experimentally, is the orally active cyclic hexapeptide PMX53, which is a potent, highly selective CD88 antagonist (Manthey et al., 2009; Woodruff et al., 2006). New formulation of PMX53 are currently being developed, and whether itself or its second-generation analogues, PMX205 have therapeutic potential are still remains to be seen (Manthey et al., 2009; Woodruff et al., 2006).

## **2.5 Rodent model for contact dermatitis**

Although there is no evidence of research that using mouse model for CCID, but there are many other types of CD experiments that use mice as a model for investigation. CBA/J, BALB/c, C57B1/6, DBA/2 mice were used in the experimental photoallergic CD which is known to be a disease in human (Maguire & Kaidbey, 1982). An improved mouse model, NC/Nga mouse, which is dermatitis-prone inbred mouse, was used for atopic dermatitis experiment (Kawakami, Yumoto, & Kawakami, 2007). BALB/c mice, in the other hand, were used to induce ACD in order to find out the effect and mechanism of aloperine (Yuan, Liu, Zhang, Wang, & Guo, 2010).

### 3.0 MATERIALS AND METHODS

#### 3.1 Mice

30 six weeks old females BALB/c mice were purchased from an outside supplier. The animals were acclimatized for a week at the Microscopic Laboratory 2. The mice experiment started at 7 weeks of age with an average body weight of  $\pm 15$  gram. The mice were divided into a group of 4; Group 1, 2, 3 and 4 with each group containing 6 mice, whereas the remaining 5 mice were used in a pilot group study. 6 mice were placed in individual plastic cages with *ad libitum* standard animal chow and water. All experimental procedures were carried out according to the UPM's Institutional Animal Care and Use Committee (IACUC) guidelines (FYP-2014/FPV.037).

#### 3.2 Chlorhexidine

4% chlorhexidine was obtained from Pharmacy Unit, Faculty of Veterinary Medicine, UPM in a form of solution. Gauze patch was chosen as the appropriate vehicle for carrying the chlorhexidine to apply to the test area. The gauze patch was held in contact in fixed position with surgical tape.

#### 3.3 Pilot study and preparation

All the mice were shaved at the dorsal part of the abdominal region of the body to reveal the bare skin around 6 cm<sup>2</sup>. 5 mice were then chosen randomly and the skin was contacted closely with the gauze patch (**Image 1**) that was fully soaked with 2 ml 4% chlorhexidine (**Image 2**) and fixed with the surgical tape (**Image 3**)

around the abdominal region (**Image 4**). The gauze patches were left for 6 hours of contact time before removing them from the mice. Every day, the mice were observed closely for any inflammatory reaction to determine the days required to induce CD to the mice's skin.

### **3.4 Induction of contact dermatitis (CD) and clinical scoring**

On day 0, 24 mice from Group 1, 2 and 3 were induced with 4% chlorhexidine for contact dermatitis. The degree of skin reaction were accessed daily and graded on a scale of 0-3 as indicated in "Magnusson and Kligman Grading Scale for the Evaluation of Challenge Patch Test Reactions" according to the OECD guideline for skin sensitisation (OECD, 1992). Each lesion was graded from 0-3: 0 described as no visible change, followed by 1 was discrete or patchy erythema, 2 was moderate and confluent erythema and 3 defined as intense erythema and swelling. In this daily observation, the lesion score were added and the grand total was calculated at the end of experiment. The mice were further exposed to 4% chlorhexidine for total of 4 days continuously with 6 hours contact time every day.

All the experiment protocols concerning the handling of the mice were according to the requirement of the IACUC.

### **3.5 Treatments**

At day 4 post chlorhexidine contact, 6 mice from each group were injected subcutaneously (s.c.) for 7 days using 1 ml syringe and 29' gauge needle as a treatment protocol: Group 1 were injected with Dexamethasone, 0.30  $\mu$ l/mouse, corticosteroid drug (Dexakel, 5 mg/ml) as positive control, Group 2 were injected

with Chlorphenamine maleate, 0.15  $\mu$ l/mouse, anti-histamine (Histamil, 10 mg/ml) and Group 3 were injected with C5a antagonist, 0.10  $\mu$ l/mouse (PMX205, 5 mg/ml).

### **3.6 Euthanasia**

At day 11, the mice were anaesthetized by using diethyl ether and humanely euthanized by doing complete exsanguination at Microscopic Laboratory 2, Faculty of Veterinary Medicine, UPM. The carcasses of the mice were disposed at Post Mortem Laboratory, Faculty of Veterinary Medicine, UPM.

### **3.7 Haematology parameters analysis**

All laboratory analyses were done at the Haematology and Clinical Biochemistry Laboratory, Faculty of Veterinary Medicine, UPM. Blood samples were collected in EDTA-anticoagulant tubes through the complete exsanguination from every mouse at day 11 using 1 ml syringe with 29' gauge needle.

### **3.8 Histopathology analysis**

Two mice from each group were selected for histopathology analysis. The skin tissue that made contact with the 4% chlorhexidine were sampled and fixed in 10% formalin solution for over 24 hours. It was followed by running through the histokinette machine and embedding into the paraffin block. 5  $\mu$ m sections were cut and fixed on the slides. Each slide was stained with haematoxylin and eosin (H&E) stain. The thickness of epidermal skin layer was measured using N-S Element D3-2 under microscope. Three measurements were taken at different area in one sample

and the data collected were evaluated and compared between each group with one way ANOVA.

### **3.9 Serum IgE ELISA analysis**

Serum of mice were collected from the blood sample after centrifugation for 15 minutes at 1000 ×g and stored under -20 °C. Before doing the assay, the serum samples were centrifuged after thawing. Then, the assay was done according to the assay procedure guided in “Cusabio Mouse IgE ELISA Kit Catalog Number CSB-E07983m” (CUSABIO Life Science, n.d.). Calculation of result was also done based on the guideline.

### **3.10 Statistical analysis**

Statistical analysis was performed using one-way ANOVA following Tukey’s test with differences were considered significant at  $p < 0.05$ .

## 4.0 Results

### 4.1 Clinical observations

There were skin reactions toward prolonged contact of 4% chlorhexidine during the experiment. Dermatology lesions were first noted after day 2 post-chlorhexidine contact with lesions of erythema and oedema at the bare skin area estimated at 2cm x 2cm size (**Image 5**). During the pilot study, after 3 days of post-chlorhexidine contact, the dermatology lesions would resolve after 4 days without any treatment. Thus, the remaining mice for the experiment were given an extra day of chlorhexidine contact.

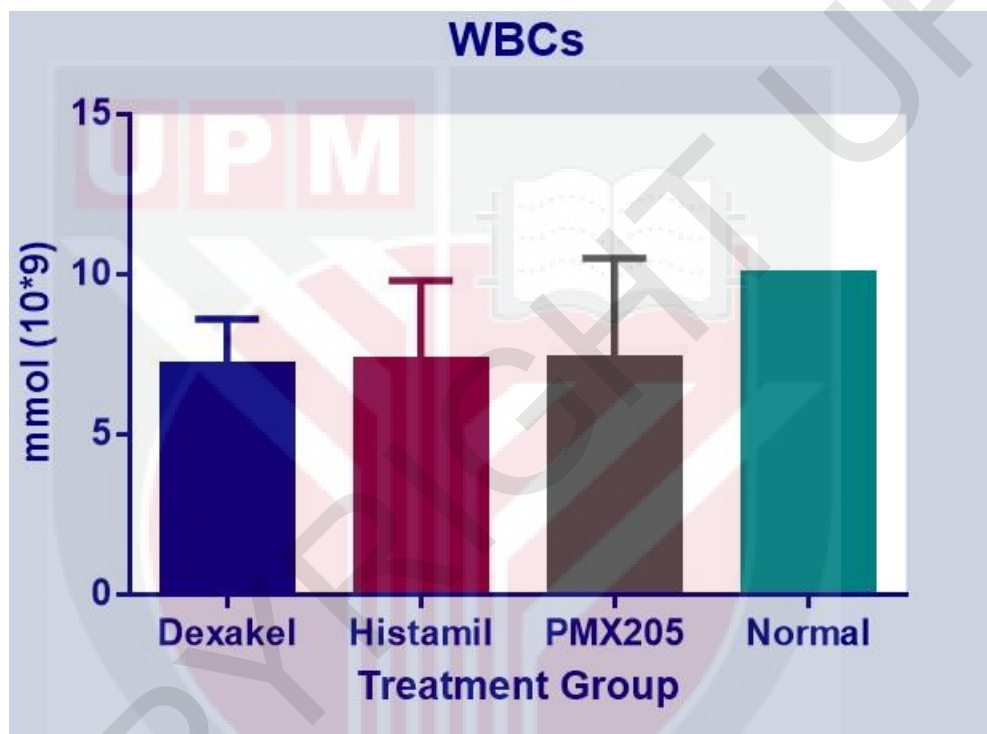
After 4 days of post-chlorhexidine contact, the skin had developed erythema and oedema, following by hardening of skin layer and crust formation after 3 days of treatment period (**Image 6**). During day 4 to day 7 of treatment period, the mice started to show sign of itchiness by biting the crusty skin area (**Image 7**).

The scores of skin lesion were tested for the assumption of normality. Review of the Shapiro-Wilk test for normality ( $SW = 0.935$ ,  $df = 30$ ,  $p = 0.066$ ) and skewness (0.155) and kurtosis (0.110) statistics suggested that normality was a reasonable assumption. The boxplot suggested a relatively normal distributional bell-shape of the scores. The Q-Q plot and histogram suggested normality was reasonable.

### 4.2 Peripheral blood evaluations

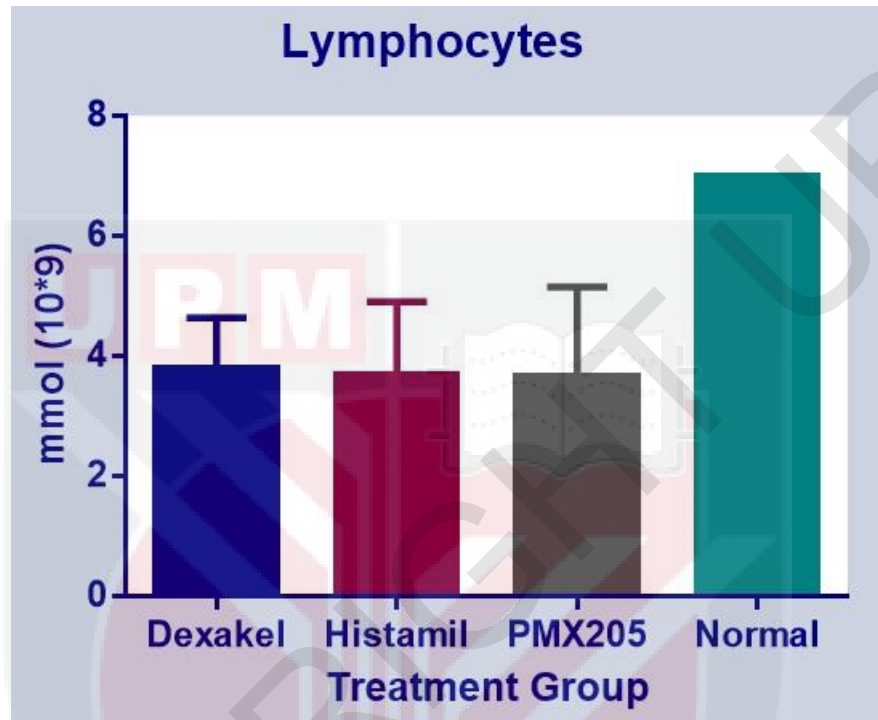
No clinical significant changes in the mean blood parameters in white blood cells (WBC) total count ( $p = 0.582$ ), neutrophils count ( $p = 0.703$ ) and lymphocytes

count ( $p = 0.167$ ) for any of the treatment group and control group (No treatment group) were recorded. There was also no significant difference in the numbers of circulating lymphocytes, neutrophils and eosinophils between groups. Basophil and mast cell were not found in these smears.



**Figure 2:** The value of total WBC count vs treated group compared with control group. No significant effects of drugs on total WBC count and between treatment groups, control group and C5a antagonist group. ( $p < 0.05$ )

**Figure 3** showed that there were no significant difference in percentage of lymphocyte count manually through thin blood smear between the control group, treatment groups, positive control group and C5a antagonist group. All the groups' result was below normal range.



**Figure 3:** The value of lymphocyte percentage vs treated group compared with control group. No significant values were recorded. ( $p < 0.05$ )

As for neutrophils, **Figure 4** shows that there were significant difference between the control groups and the treated groups. Yet, in **Figure 5**, eosinophil parameter again showed no significant difference between each groups.

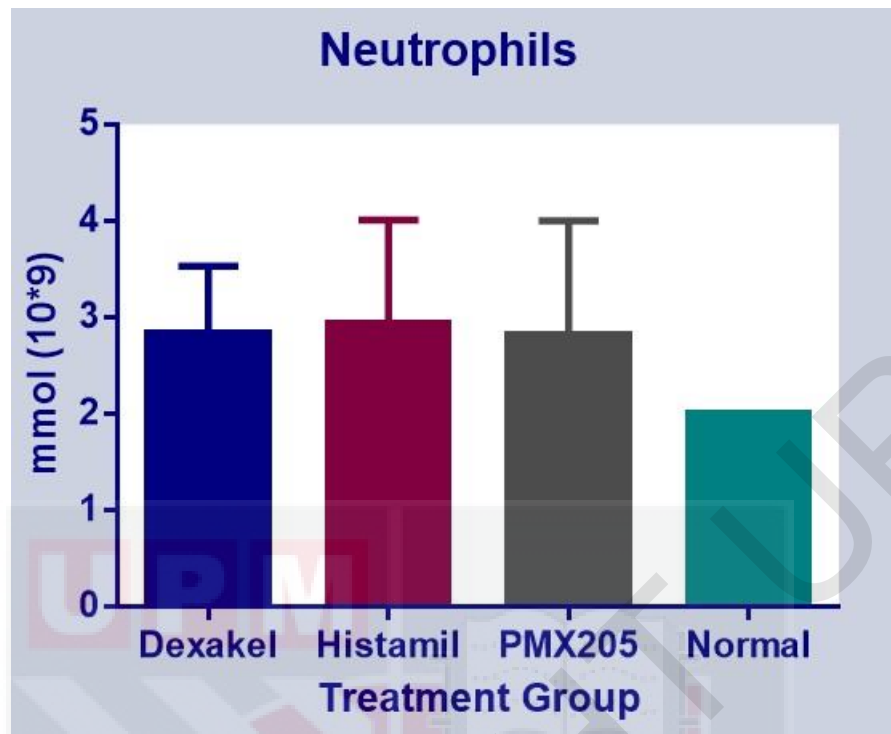


Figure 4: The value of neutrophil percentage vs treated group compared with control group. Significant value was recorded. ( $p < 0.05$ )

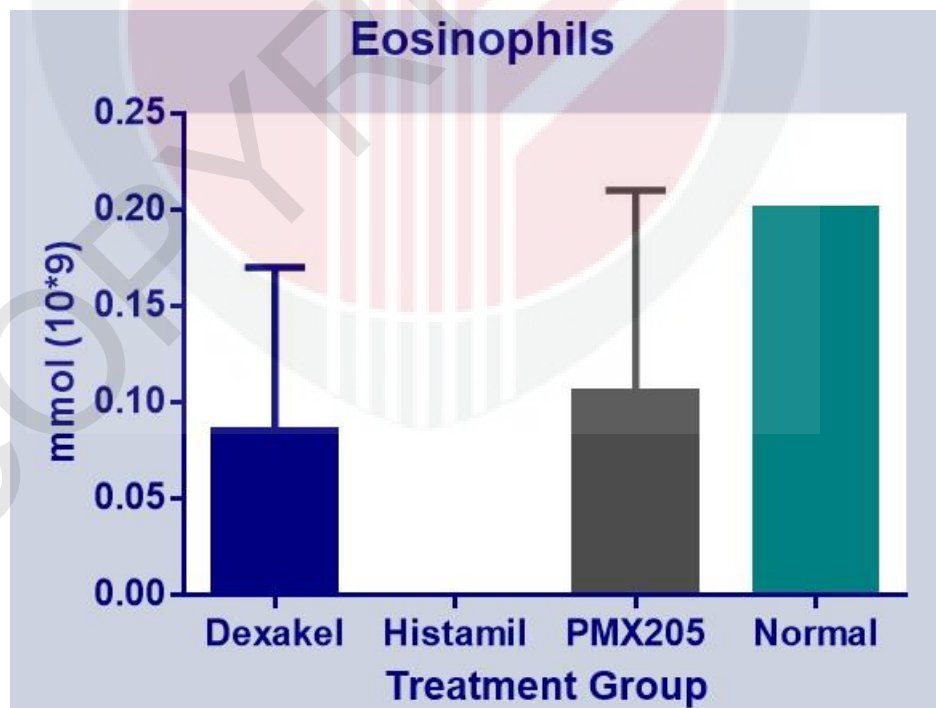


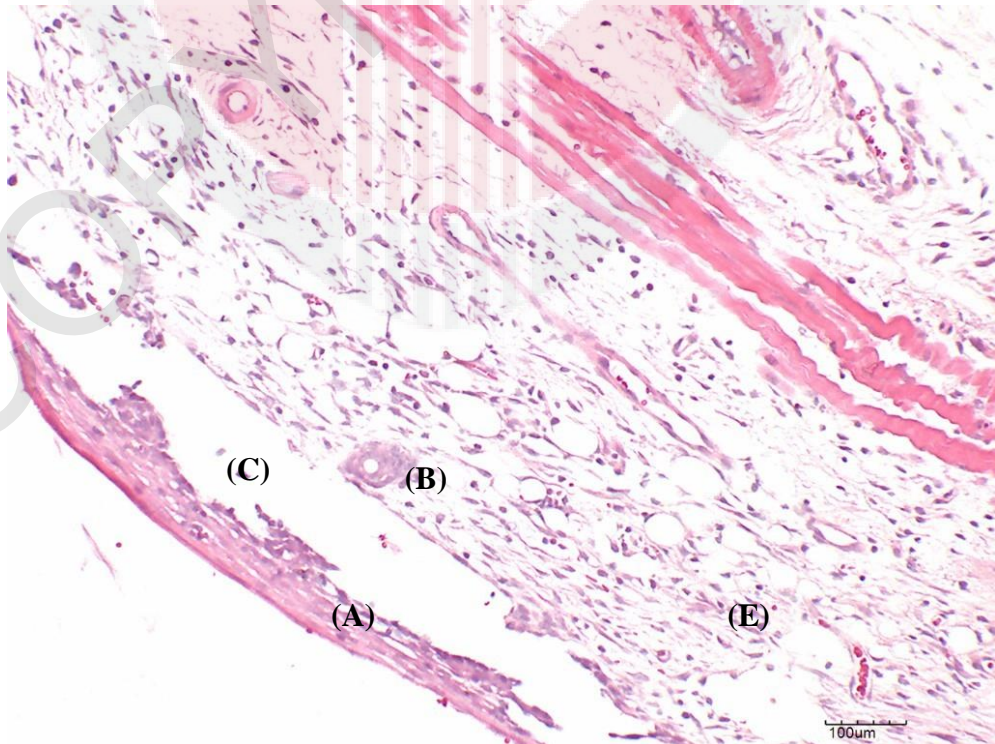
Figure 5: The value of eosinophil percentage vs treated group compared with control group. No significant value was recorded. ( $p < 0.05$ )

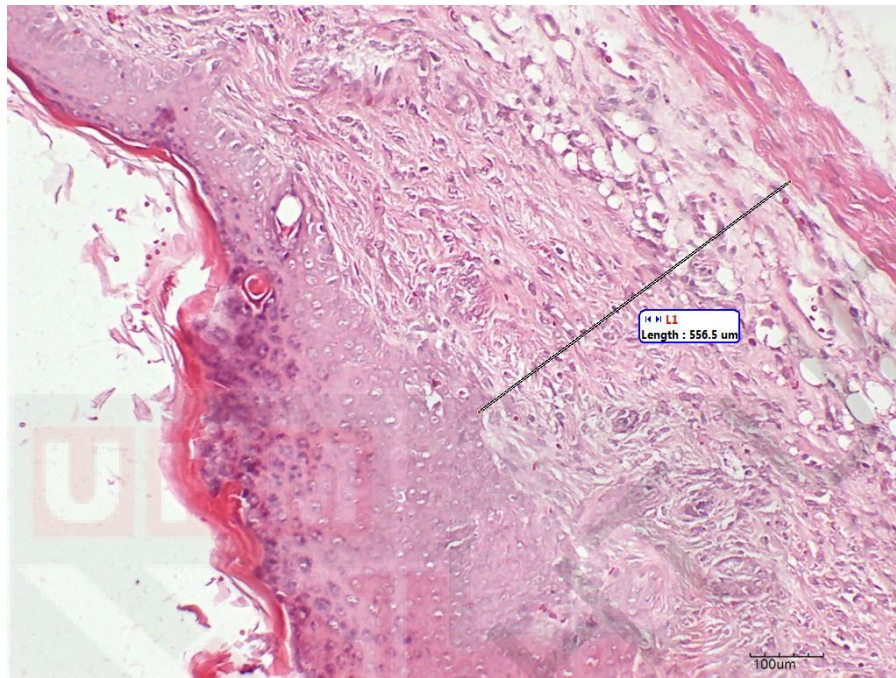
### 4.3 Histopathological lesions of the skin

All groups showed histopathological changes in the epidermis and dermis layer whereby the findings were similar in Frosch, Menné & Lepoittevin, 2006 manifested by parakeratosis of the epidermis layer, necrosis of hair follicles, separation of epidermal and dermal layers, spongiosis with weakening of the tissue structure and infiltration of inflammatory cells (**Figure 6.1 & 6.2**). The severity of the inflammation was measured by comparing the thickness of the dermis layer as shown in some of the examples in **Figure 7, 8, 9, and 10**. There was significant difference between the treatment groups as determined by one-way ANOVA ( $F(5,30) = 21.838, p = 0.000$ ) (**Table 15**). A Tukey post-hoc test (**Table 16**) revealed that there were statistically significant difference by comparing C5a antagonist group ( $428.10 \pm 92.43 \mu\text{m}$ ) with no treatment group ( $1061.65 \pm 272.54 \mu\text{m}, p = 0.000$ ) and normal group ( $756.02 \pm 148.06 \mu\text{m}, p = 0.007$ ). There were no statistically significant differences between the C5a antagonist with Dexakel ( $560.58 \pm 73.51 \mu\text{m}, p = 0.638$ ), and Histamil ( $241.53 \pm 98.45 \mu\text{m}, p = 0.277$ ) groups. However, there was significant difference between the thickness of skin in Dexakel treatment group and Histamil group ( $p = 0.01$ ).

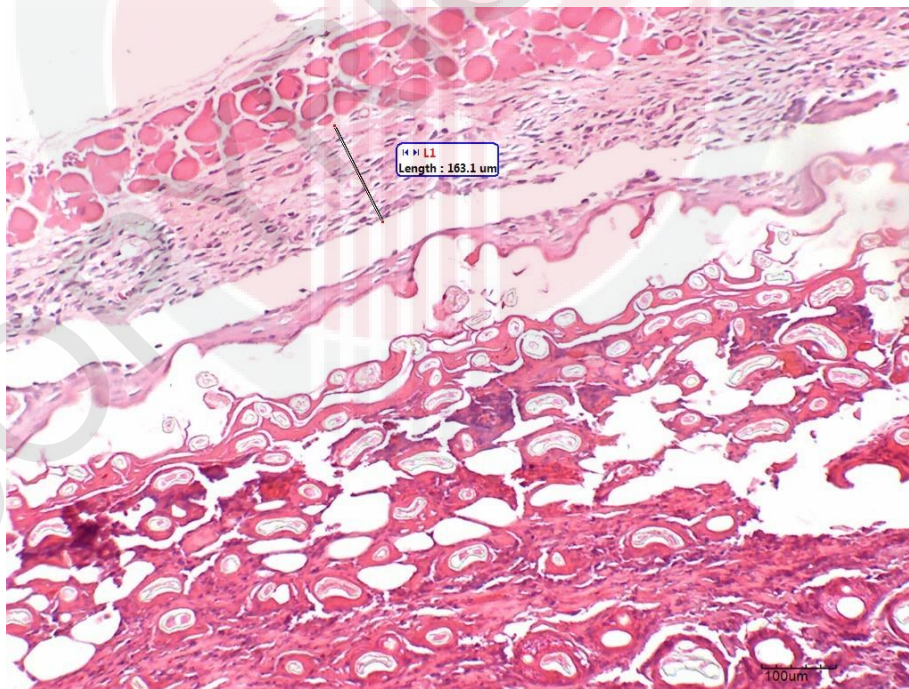


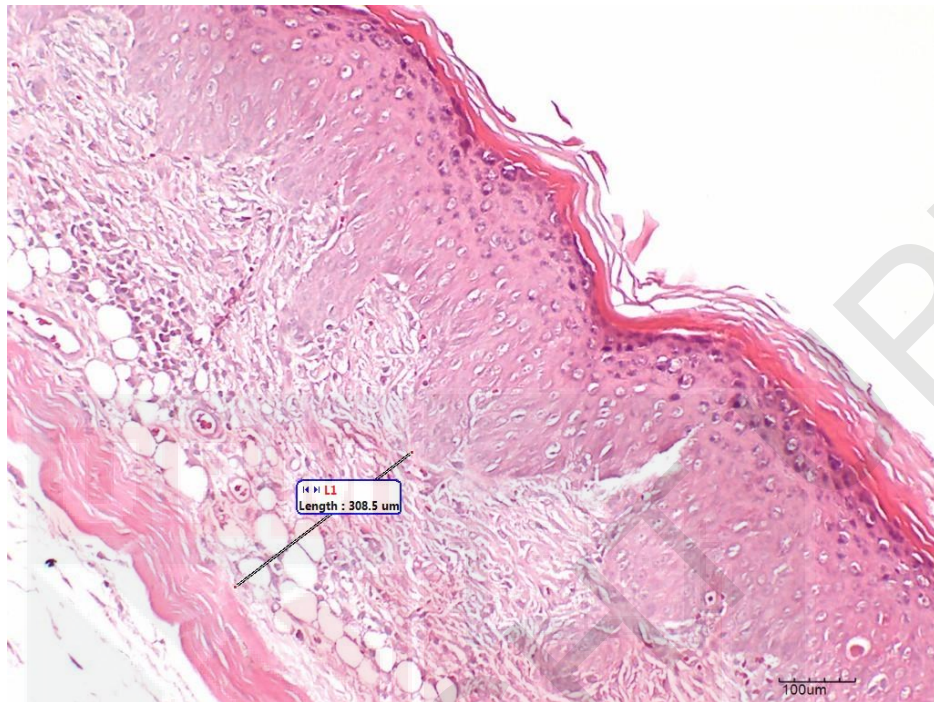
**Figure 6.1 (up) & 6.2 (below):** Histopathology changes in mouse skin layer in negative control group (No treatment group); (A) parakeratosis of the epidermis layer, (B) necrosis of hair follicles, (C) separation of epidermal and dermal layers, (D) spongiosis with weakening of the tissue structure and (E) infiltration of inflammatory cells.



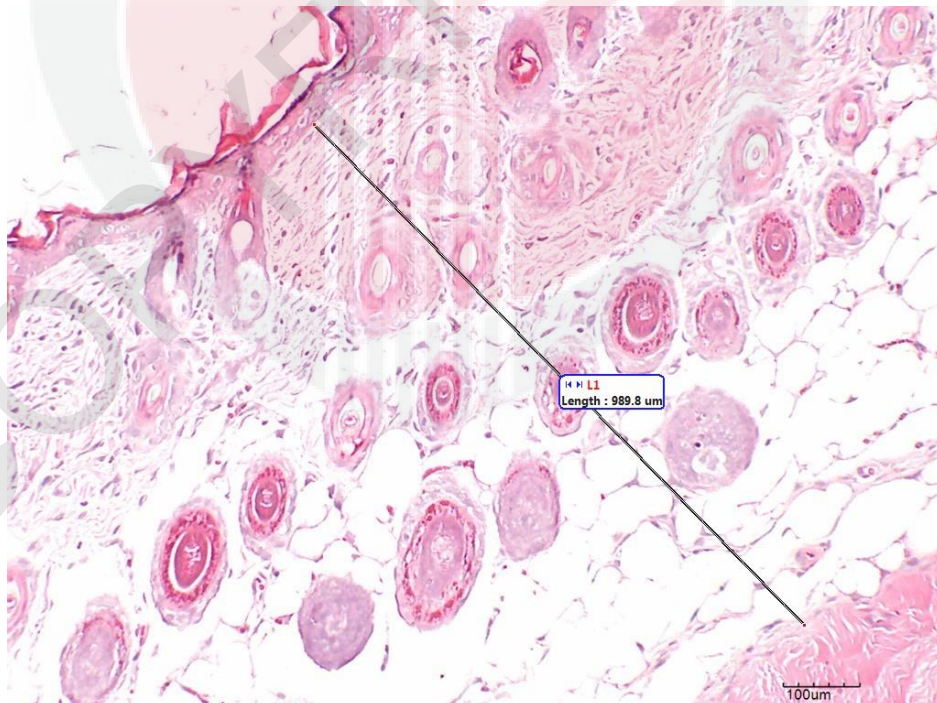


**Figure 7 (up) & Figure 8 (below): One measurement of the dermis layer in Dexakel and Histmil group respectively.**



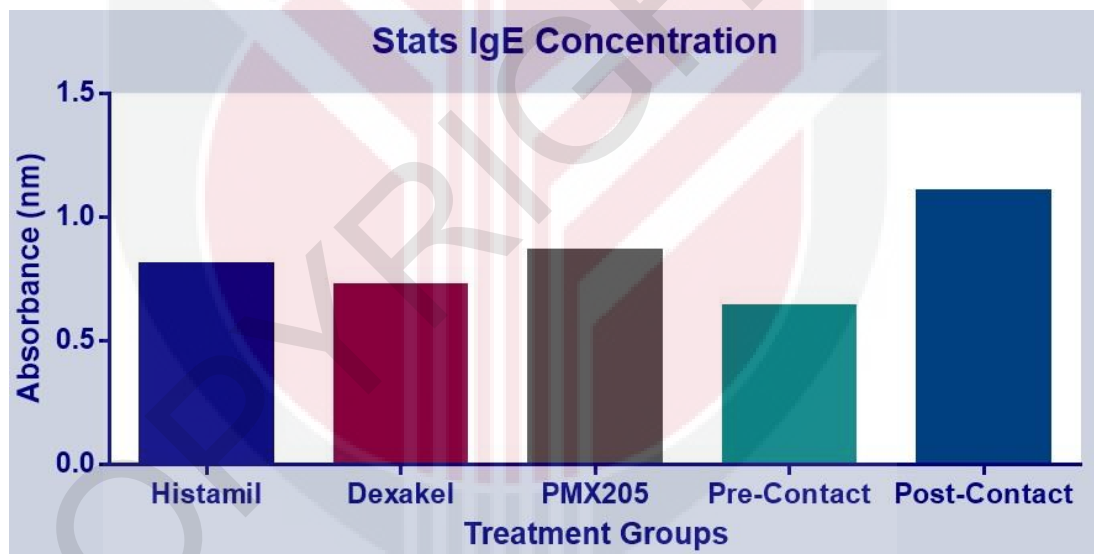


**Figure 9 (up) & Figure 10 (below): One measurement of the dermis layer in C5a antagonist and normal group respectively.**



#### 4.4 Serum IgE ELISA reading

The absorbance level of the serum of the blood was measured and the average absorbance values were collected for one-way ANOVA analysis. According to **Figure 11**, there were significant differences between the serum IgE level in the blood serum of the treatment groups and the control group ( $F(4,10) = 2.306 \times 10^9$ ,  $p = 0.000$ ). A Tukey post-hoc test revealed that there were statistically significant differences by comparing C5a antagonist group with no treatment group, normal group, Dexakel, and Histamil groups.



**Figure 11:** The value of serum IgE concentration by measuring average absorbance vs treated group compared with positive (post-contact) and negative (pre-contact) control groups. There were significant values recorded based on the graph shown.

## 5.0 Discussion

For many years, chlorhexidine-containing products have also been used in other areas of medicine because of chlorhexidine's beneficial effects as a topical antimicrobial agent. These products include antiseptic skin creams and disinfectants used to prepare the skin for surgical procedures. In the early 90's, chlorhexidine have also began to be incorporated into the composition of medical devices including intravenous catheters, topical antimicrobial skin dressings and implanted antimicrobial surgical mesh (Food and Drug Administration, 1998). From its use in all of these medical applications, it has become apparent that chlorhexidine has the potential to produce hypersensitivity reactions (Pemberton & Gibson, 2012). The Type IV hypersensitivity reaction of CD to chlorhexidine has been reported in both adults and children with confirmation by patch testing in many cases (Goon et al., 2004; Le Corre, Barbarot, Frot, & Milpied, 2010; Liippo, Kousa, & Lammintausta, 2011). This delayed type of hypersensitivity has most frequently followed the use of chlorhexidine-containing topical medicinal creams used to treat inflamed skin (Pemberton & Gibson, 2012). However, in other reports of possible hypersensitivity relating to chlorhexidine-containing products, it is sometimes unclear as to which type of immunological response is actually occurring (Yaacob & Jalil, 1986).

Chlorhexidine is a commonly used antiseptic agent in health care, yet ACD is seldom reported (Goon et al., 2004; Toholka & Nixon, 2013) and therefore, its role as an allergen is often mis-diagnosed (Calogiuri et al., 2013). Studies have shown that chlorhexidine is an important allergen in health-care workers and should not be overlooked when they are presented with hand dermatitis (Liippo et al., 2011;

Toholka & Nixon, 2013). In addition, patients may become sensitised through other exposures to chlorhexidine which may be non-occupational (Toholka & Nixon, 2013). Thus, it is important for practitioners to raise their awareness of ACD to chlorhexidine, as well as the healthcare workers in medical field, who are readily exposed to chlorhexidine-containing skin cleansers and other products.

Appropriate management of patients with ACD or ICD involves both treatment of symptoms and avoidance of continuous exposures with the allergens (Cohen & Heidary, 2004). The introduction of topical corticosteroids to treat for contact dermatitis, and other eczematous skin diseases has started in the 50's (Cohen & Heidary, 2004; Wollenberg & Bieber, 2001). Effectiveness of topical steroids in the treatment of ACD is well documented. In a double-blinded study, 0.05% fluticasone propionate was effective in reducing nickel-induced contact allergy (Hachem et al., 2002). Although corticosteroids have been shown in other studies to have a beneficial effect in ACD (Queille-Roussel, Duteil, Padilla, Poncet, & Czernielewski, 1990), their role in ICD remains controversial. Triamcinolone acetonide 0.05% cream was evaluated in ICD induced by repetitive short exposure to a low molarity sodium dodecyl sulfate (SDS) solution (Le, De Mon, Schalkwijk, & van der Valk, 1997). The reports on the effects of corticosteroids on ICD are far from unanimous. Levin et al., 2001 found corticosteroids to be ineffective in treating surfactant-induced irritant dermatitis when compared with the vehicle and the untreated control (Levin et al., 2001). Oral and topical corticosteroids are generally well tolerated when used short term. However, long-term and widespread use of

topical steroid use can result in cutaneous atrophy, hirsutism, folliculitis, and systemic absorption (Cohen & Heidary, 2004).

Anti-histamine drugs, which are also known as histamine inhibitors, are commonly used to treat allergic inflammatory diseases, such as atopic dermatitis (Ohsawa & Hirasawa, 2012). Inflamed skin contains mast cells, eosinophils, and Th2 cells and exhibits histological abnormalities such as scaling, crusting and lichenoid papules (Leung, Boguniewicz, Howell, Nomura, & Hamid, 2004; Novak, Bieber, & Leung, 2003). The patient would show clinical signs characterized by intense pruritus, chronic eczematous plaques and relapsing inflammation induced by repeated exposure to an antigen (Ohsawa & Hirasawa, 2012). There are four types of histamine receptors that have been identified, which are histamine H1 receptor (H1R), histamine H2 receptor (H2R), histamine H3 receptor (H3R), and histamine H4 receptor (H4R), to be targeted for its antagonism effect on behalf of histamine inhibition (Hirasawa et al., 2009). In Ohsawa and Hirasawa's study (Ohsawa & Hirasawa, 2012), they have revealed that the H1R antagonist and H4R antagonist could improve the skin inflammation in a model of chronic allergic dermatitis established in NC/Nga mice. Suwa et al. (Suwa, Yamaura, Oda, Namiki, & Ueno, 2011) also reported the therapeutic effects of H4R antagonist in chronic dermatitis. Thus, histamine is involved in the development of dermatitis in a model of chronic allergic dermatitis and treatment with anti-histamine is possible due to the potentiated anti-pruritic and anti-inflammatory effects, which is equivalent to the pharmaceutical benefit of corticosteroids (Ohsawa & Hirasawa, 2012). However, only certain combinations of histamine receptor antagonists could give such a

satisfying pharmaceutical result as corticosteroids since solely depending on one type of the histamine receptor antagonist is not that effective. (Hirasawa et al., 2009; Ohsawa & Hirasawa, 2012). That is the reason why an alternative drug was considered for the treatment of dermatitis, especially ACD in this study, by modulating the complement receptor, in order to block the production of C5a, which served as a pro-inflammatory mediator that cause inflammation in CD (Czermak et al., 1998; Esser, 1994; Guo & Ward, 2005; Kirschfink, 1997; Matis & Rollins, 1995).

In this study, we have successfully proved that chlorhexidine would induce CD in mouse model after 6 hours of close contact for 4 days respectively by looking at the development of clinical signs such as erythema and oedema, followed by hardening of skin layer and crust formation and lastly pruritic sign.

However, the blood parameter for total WBC count, lymphocyte and eosinophils by their average mean were within normal range in the post CICD and post treatment for 7 days except for the neutrophils, which is significantly above normal range. This could be due to the natural immune response toward the antigen and development of leukocyte infiltration (Detmar et al., 1998; Wilkerson et al., 2004). When the skin first contacted with the allergen, or haptens, it is able to induce sensitization of skin via two properties: firstly, through activation of skin innate immunity with its pro-inflammatory properties and secondly, through modification of self-proteins that allows expression of new antigenic determinants by their binding to amino-acid residues. This sensitization steps can last up to 5 to 7 days in the mouse before the massive infiltration of leucocytes (Saint-Mezard et al., 2004), whereas in this study, the mice were sensitised for 4 days only and immediately given treatment for 7 days. Thus, there was not enough time for the development and

infiltration of total WBC count, lymphocyte and eosinophils except for the neutrophils. Furthermore, this study was a preliminary study, so references pertaining in the use of corticosteroid, anti-histamine and C5a antagonist in treating CICD were limited. Therefore, it could also be suggested that the drugs might react towards the inflammatory reactions but did not alter the normal blood parameters. That might explain why the treated mice groups had lower blood parameter as compared to the control mice group.

As for the histopathological changes, some of the lesions observed in the skin samples were mentioned in the summary of the predominant histopathological features induced by selected chemical test in (Frosch, Menné & Lepoittevin, 2006). Damage could be seen at all levels of the epidermis, as well as the overwhelming cellular destruction if applied at high concentration (Frosch, Menné & Lepoittevin, 2006). In this study, epidermal layers in some of the samples were disintegrated due to increased fragility at the lesion area as well as due to technical error during paraffin wax cutting process. Thus, dermis layer was chosen to be measured in order to acquire equitable and consistent results.

Formation of parakeratosis of the epidermal layer is a feature indicative of enhanced keratinocyte proliferation, which further confirmed a significant physiological response to chlorhexidine (Frosch, Menné & Lepoittevin, 2006; (Willis, Stephens, & Wilkinson, 1992). Necrosis of hair follicles, separation of epidermal and dermal layers, spongiosis with weakening of the tissue structure are also part of the significant pathogenesis of CD (Willis, Stephens, & Wilkinson, 1989). The infiltration of inflammatory cells is suggestive of inflammatory reaction towards the allergen. By comparing the thickness of the dermis layer between the treatment

groups and the control groups, we could conclude that the C5a antagonist could reduce the inflammation of the skin as comparable to corticosteroid and anti-histamine.

In this study, serum IgE in the blood of the mice has significant difference between the treatment groups and the negative control group. This result has supported the theory that chlorhexidine being a potential allergen that could induced ACD. Other study has also proven that chlorhexidine could induce ACD through measuring the specific IgE and histamine release to confirm an IgE-mediated mechanism for allergy to chlorhexidine (Garvey et al., 2007). It has shown that measuring IgE levels are beneficial for diagnosing allergy to chlorhexidine (Garvey et al., 2007).

## **6.0 Conclusion**

Chlorhexidine indeed will induce ACD in animals especially in mice model. In this study, the use of anti-histamine is the most beneficial towards the treatment for CCID and effects of C5a antagonist provides an alternative treatment for CCID.

## 7.0 Recommendation

Study on the effects of C5aR antagonism on CICD in mouse model have never been done before and therefore, further studies warranted. Besides using BALB/c strain as a model, CBA/J, C57B1/6, DBA/2 or NC/Nga mice strains can also be used as a model for induction of ACD and atopic dermatitis (Kawakami et al., 2007; Maguire & Kaidbey, 1982). The duration of chlorhexidine contact period should be prolonged for at least 20-22 days based on (OECD, 1992) in order to have first induction period for 5-7 days, second induction period for 12-14 days and challenge period for 24 hours.

Further studies need to be done to understand more about the inflammatory response of chlorhexidine as allergen, early generation of C5a during the cellular recruitment and subsequent pathology induced by chemokines and other secondary mediators by monitoring other parameters such as interleukin or binding affinity of complement receptor (Bhatia et al., 2001; Manthey et al., 2009). Further identification of cellular pathology through histopathology changes in mice skin can be done by having better visualization of keratinocytes with high resolution light microscopy in Toluidine blue-stain or immunoperoxidase labelling with monoclonal antibody (Frosch, Menné & Lepoittevin, 2006).

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## 9.0 Appendices



**Image 1: 2 x 2 cm square gauze.**

**Image 2: 2ml 4% chlorhexidine pumped on the square gauze that fixed on a 6cm long surgical tape.**



**Image 3: Micropore surgical tape used for the purpose of vehicle to carry the chlorhexidine.**

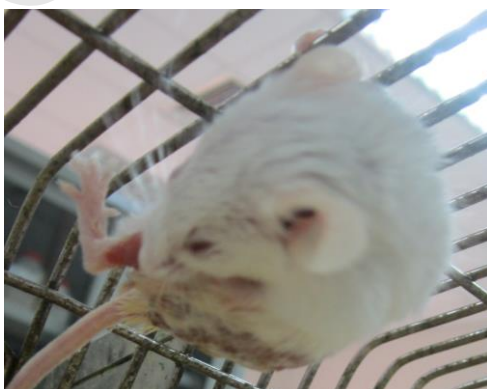
**Image 4: Body of mouse taped with chlorhexidine-loaded gauze and fixed for 6 hours contact time.**





**Image 5: Lesions of erythema and oedema at the bare skin area of its dorsal back with estimation of 2cm x 2cm size.**

**Image 6: Clinical signs of hardening of skin layer and crust formation after 3 days of treatment period.**



**Image 7: Sign of itchiness by biting the crusty skin area.**