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DETERMINATION OF PERMEATION PATHWAY OF TETRACYCLINE INTO THE PIG'S EAR SKIN

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ABSTRACT

The important roles of hair follicles as permeation pathway of topically applied drugs have gained recent attentions in several fields of research especially dermatological sciences. Hair follicles are increasingly considered to represent as one of drug delivery routes through the stratum corneum into deeper skin layers. This study was conducted to confirm the penetration pathway of Tetracycline into pig's ear skin through comparing the concentration of Tetracycline between non-plugged pig's ear skin and plugged pig's ear skin which were done through hair follicular blocking method by using silicone. The findings from this study showed that the permeation of tetracycline through non-plugged pig's ear skin is higher compared to plugged pig's ear skin. As such, the maximum concentration of tetracycline permeated through plugged pig's ear skin was generally lower than non-plugged pig's ear skin. Statistical analysis by using t-test showed that P-value from the cumulative amount of permeation of Tetracycline in both non-plugged and plugged pig's ear skin is $P<0.05$. Thus, the difference between cumulative amount of concentration of Tetracycline for duration of 24 hours study between non-plugged and plugged pig's ear skin are considered to be statistically significant as $P<0.05$. Thus, the present findings clearly suggest that the permeation pathway of tetracycline drug into pig's ear skin is through hair follicles.

KEYWORDS: Tetracycline, Hair follicles, Pig's ear skin, Permeation pathway.

INTRODUCTION

The skin is the largest human organ. It has an important function of protecting the body from the surrounding environment, particularly being an efficient barrier to exogenous molecules (Godin and Touitou, 2007). The stratum corneum is the outermost skin layer and represents the barrier of the human organism to the environment. It consists of cornified cells known as corneocytes which are surrounded by lipids. As such, in order to penetrate into or through the skin, topically applied substances, such as medical or cosmetics products must overcome this barrier (Lademann *et al.,* 2008).

Skin penetration however, is necessary to particular circumstances for example, in the dermatological treatment which necessitate the delivery of drug to the skin and through skin by transdermal patches as well as in cosmetic area which involved skin care and protection. Besides, skin allowing sustained drug delivery to the blood circulatory system as an alternative route for drug administration while avoiding several side effects of oral and parenteral administration (Bolzinger *et al.,* 2012).

There are two general options for drugs substances to permeate the stratum corneum which are trans-epidermal route and via transappendageal route. The trans-epidermal route can be further divided into transcellular and intercellular route. Through transcellular route, the drug has to directly passing through both the lipid structures of the stratum corneum and the cytoplasm of the dead keratinocytes. This is the direct route for drug penetration across the skin and the shortest route for the drug substances. However, the substances encounter significant resistance to permeation because they have to cross both lipophilic and hydrophilic structures of the skin (Trommer and Neubert; 2006).

Penetration pathway via intercellular route, on the other hand is the common route for drugs substances as the permeant overcomes the stratum corneum by passing between the corneocytes. The intercellular route is certainly the major penetration pathway but penetration through transappendageal routes may be important in specific cases (Bolzinger *et al.,* 2012). The transappendageal routes are also known as the shunt routes involve permeation through the sweat glands and across the hair follicles with their associated sebaceous glands. It opens directly into the environment at the skin surface (Leyden, 2003).

In particular, skin provides an alternative route for drug administration, allowing sustained drug delivery to the blood circulatory system and providing greater comfort for the patient, while avoiding several side effects of oral and parenteral administration. However, the huge barrier function of the stratum corneum makes topical drug delivery challenging despite several advantages such as a large available surface area for penetration of the drug and relatively low enzymatic degradation of drug through topical route. Percutaneous or dermal absorption describes the passage of compound across the skin. The process is divided into three steps; penetration which involve the entry of a substance into a particular skin layer, permeation pathway in which it involves the penetration layer into another layer and finally resorption, the uptake into the vascular system (Bolzinger *et al.*, 2012).

Penetration of topically applied compounds may occur through stratum corneum as well as through skin appendages including sweat glands and hair follicles. The three main permeation routes through the stratum corneum are the intercellular, intracellular and follicular pathways. The structure of stratum corneum may be represented as a ‘brick and mortar wall’. Such a model serves as the basis of predicting the permeability of the stratum corneum to penetrant molecules and taken into account passive diffusion driven by the concentration gradient of penetrant molecules (Bolzinger *et al.*, 2012).

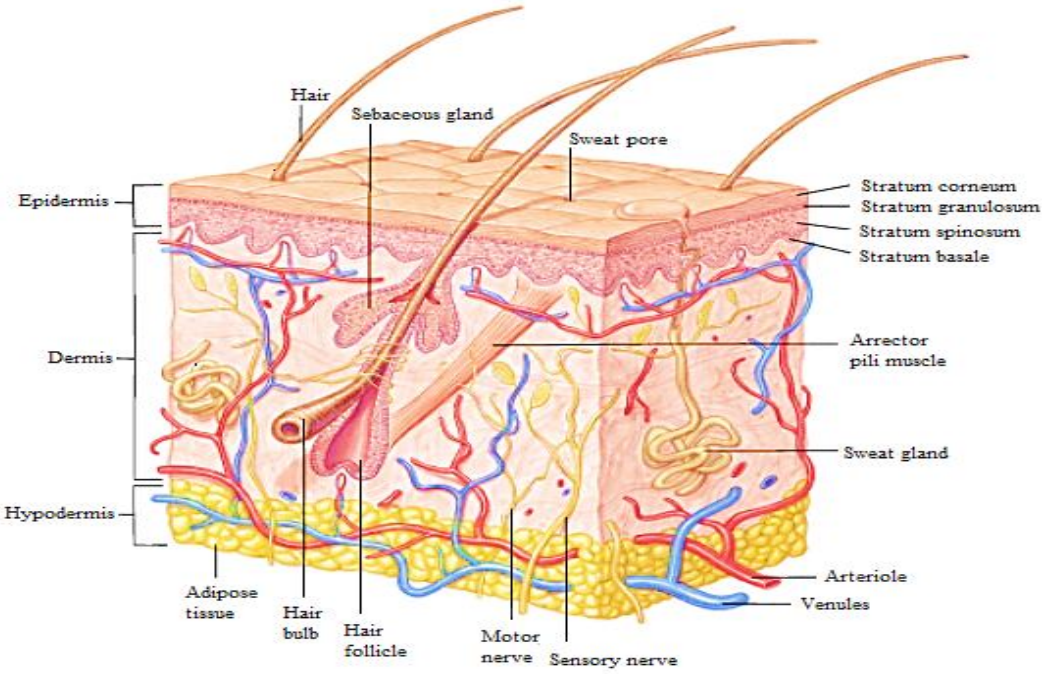

Figure 1: Structure of Skin

Nowadays, hair follicles have attracted much attention in several field of research. They are increasingly considered to be relevant drug targets and to represent drug delivery routes through the stratum corneum into deeper skin layers. The penetrating substance with its associated vehicle are not the only aspect that determines the effectiveness of follicular or even trans-follicular penetration but it also depends on the activity status of the hair follicle. Follicular targeting offers opportunities in the treatment of hair follicle-associated disease such as acne which ideally deliver directly topical drug substances to specific compartments and cell populations within the hair follicles (Patzelt *et al.*, 2008).

Multiple studies suggest that the follicular penetration route may be especially relevant for hydrophilic and high molecular weight molecules as well as by particle-based drug delivery system. The follicular route seems to promote permeability at the beginning of skin absorption while it is overrules by the other higher capacity pathways at later times in the transport process for several compounds e.g. caffeine, hydrocortisone and testosterone. In the context of the absorption of nanoparticles, the follicles have received much higher attention. It is commonly agreed that in non-damages skin the hair follicles play a central role in the penetration of solid nanoparticles (Schneider *et al.*, 2009).

The important role of hair follicles as penetration and reservoir studies for topically applied compounds has been validated in numerous animal studies as well as humans as Knorr *et al.*, 2011 has claimed that the follicle possesses distinct characteristic which favours penetration. Follicle features tight junctions in the lower part of the follicles which prevents particles from easily invading into the living cells. Instead, once entered into the follicles openings particles will be stored there until being cleared by hair growth or sebum production.

The hair follicle represents the in vagination of the epidermis extending deep into the dermis, thus providing a greater actual area for potential absorption although the follicular orifices generally occupy no more than about 0.1% of the total skin surface area. Under some circumstances, all these features listed including extensive capillary network of hair follicle allow the follicles to function as rapid transport shunts that permit topically applied drugs to bypass the continuous stratum corneum and readily reach either the viable skin layers or the systemic circulation (Meidan *et al.*, 2005).

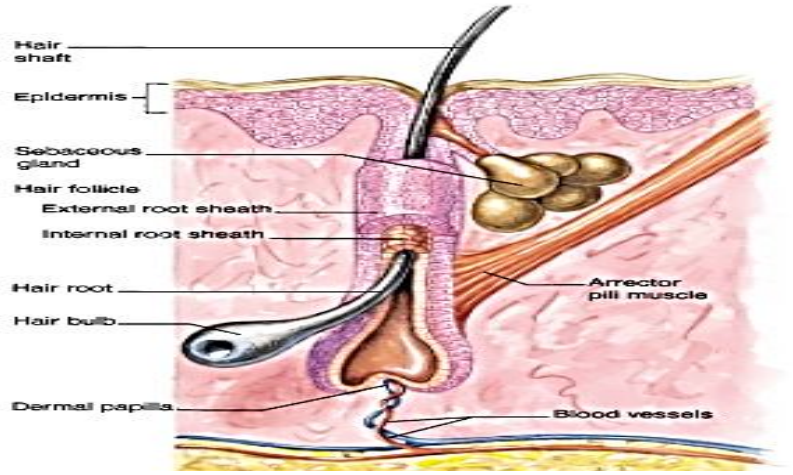

Figure 2: Anatomical structure of hair follicle

Acne vulgaris is a chronic inflammatory disease of the pilosebaceous unit, comprises of hair follicles in the skin that are associated with an oil gland. Acne results from androgen-induced increase sebum production, altered keratinisation, inflammation and bacterial colonisation of hair follicles on the face, neck, chest and back by *Propionibacterium acnes (P acnes)* in which the distribution of acne corresponds to the highest density of pilosebaceous units (Hsieh and Chen, 2011). The clinical features of acne include seborrhoea (excess grease), non-inflammatory lesions (open and closed comedones), inflammatory lesions (papules and pustules) and various degrees of scarring (Williams *et al.,* 2011).

Acne vulgaris is a common skin condition with substantial burden both in terms of cutaneous and psychological disease. Studies suggest that the emotional impact of acne is comparable to that experienced by patients with systemic disease, like diabetes and epilepsy. Acne approximately affecting 85% of teenagers and it is most common in adolescents. The average age of onset is 11 and 12 years in girls and boys respectively. It is well known that adult acne is more common in women than men (Knutsen-Larson *et al.,* 2012). Majority of the patients have both Non-inflammatory and inflammatory acne lesions. Non-inflammatory lesions predominate in mild cases with occasional papules or pustules. In moderate cases, it exhibits more papules and pustules and nodular lesion dominate the most severe cases of acne (Leyden, 2003).

Antibiotic therapy is a long-standing practice in acne treatment. Although acne is not an infection, the responsible stimulus for inflammation in acne is *P acnes*, and a reduction in *P acnes* populations is usually accompanied by clinical improvement of the acne (Webster *et al.,* 2008). The practice is generally considered to be safe and effective as many patients'

acne is effectively treated with the use of long-term antibiotic regimens. *P acnes* is the predominant inhabitant of skin regions rich in sebaceous glands and in healthy skin, it appears to be an exclusive resident within pilosebaceous follicles (Eady *et al.*, 2013). Topical antibiotics may be used to treat mild to moderate acne. Systemic antibiotics are indicated when acne is moderate to severe or if disease manifestations are producing marked psychosocial stress for patients.

The purpose of this treatment modality is to decrease the presence of *P acnes* on the skin surface and within the pilosebaceous unit. Development of *P acnes* resistance has been associated with widespread and long-term use of antibiotics (Knutsen-Larson *et al.*, 2012). The mechanism of action by which antibiotics work in acne varies with the drug. Some medications may have either anti-inflammatory or antibacterial properties, whereas other medications possess both properties.

For example, benzoyl peroxide and the macrolides antibiotics; clindamycin and erythromycin work mainly by reducing *P acnes* levels, but they have minimal anti-inflammatory activity. In contrast, the tetracycline family of antibiotics has both potent anti-inflammatory and antibacterial properties (Webster *et al.*, 2008). Tetracycline has shown to have broad spectrum agents as tetracycline exhibiting activity against gram (+) and gram (-). However, tetracycline is not widely used to treat acne topically as the most commonly used agents are topical clindamycin and erythromycin.

Besides, oral tetracycline is usually reserved for management of severe cases of acne. Therefore, further research is needed to prove topical tetracycline can be effectively used same like clindamycin and erythromycin in acne. A new effective way to suppress the growth of *P acnes* is to target the environment in which it thrives (Eady *et al.*, 2013). As such, in this situation it is involve with targeting pilosebaceous unit so as to reduce the development of *P acnes* resistance associated with long-term of antibiotics and become the focus of study in this research.

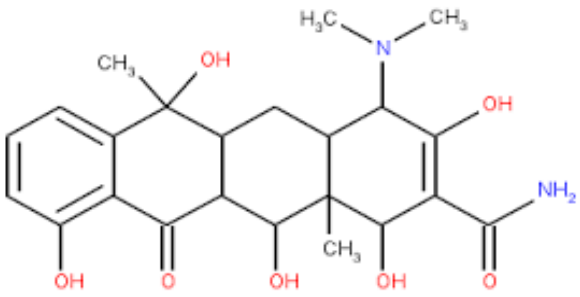

Figure 3 Structure of Tetracycline

Hence, the aim of the present study was to confirm permeation pathway of tetracycline through follicular route. The further, objective was to compare drug concentration of tetracycline between plugged and non-plugged skin.

MATERIALS AND METHOD

Materials

Tetracycline powder were obtained from Pharmaniaga Manufacturing Berhad (Bangi, Selangor, Malaysia), Silicone grease were from Synco Chemical Co., Ltd. (Bohemia, NY, U.S.A.), Nile red colouring agent were purchased from PPB Group Berhad, (Kuala Lumpur, Malaysia), Pig's ear skin were purchased from Pig Farm (Kuala Selangor, Selangor, Malaysia), Potassium dihydrogen phosphate, KH_2PO_4, Sodium chloride, NaCl, Disodium phosphate, Na_2HPO_4, Hydrochloride acid, HCl and Methanol, MeOH were from Sigma Aldrich Co., Ltd. (St. Louis, MO, U.S.A.).

Equipments

Vertical Diffusion Cell were from PermeGear Inc. (Hellertown, USA), Sonicator was from Restek (Bellefonte, PA, USA), UV Spectrophotometer was from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), Water bath TC-502D-230 was obtained from Brookfield Engineering Laboratories, Inc. (Middleborough, MA, USA), Analytical Balance GR-200 was purchased from A&D Company Ltd. (Toshimaku, Tokyo, Japan), pH meter was from Hanna Instrument Ltd. (Woonsocket, RI, USA). Eppendorf tubes were purchased from Sigma Aldrich Co., Ltd. (St. Louis, MO, USA), Magnifying glass was purchased from Lik Soon Sdn Bhd (Kuala Lumpur, Malaysia), Toothpicks were from Kim Ban Marketing Sdn Bhd (Shah Alam, Selangor, Malaysia), Shaver Series 500 was purchased from Koninklijke Philips Electronics N.V. (Kuala Lumpur, Malaysia).

Methods

Preparation of Excised Pig's Ear Skin

The pig's ear skin was obtained freshly without any pre-treatment from the slaughtered house and was excised immediately prior before use. The pig's ear skin was carefully cleaned with distilled water to remove any dirt which entrapped on the surface of the skin. The pig's ear skin was excised into circular piece which follows the size of Vertical Diffusion Cell. Then, the fats as well as blood vessels under the pig's ear skin were removed and only the stratum corneum and the epidermis or dermis of the pig's ear skin was remained for the experiments.

Preparation of Plugging Agent

0.2g of silicone grease was mixed well with 0.25mg of nile red colouring agent prior before use for plugging the hair follicles of pig's ear skin.

Follicular Blocking of Plugged Pig's Ear Skin

The pig's ear skin hair was removed by using shaver prior to preparation of excised pig's ear skin to obtain clean shaved area of pig's ear skin. By using magnifying glass, follicular blocking on the pig's ear skin was done by using hair follicles plugging agent previously prepared using toothpicks with the diameter of 0.5mm. The effective permeation area that has been plugged was estimated to be 1.77cm^2. Such that, the average number of hair follicles within the effective permeation area that was plugged by using plugging agent is between 50 to 60 follicles.

Preparation of Tetracycline Stock Solution

Tetracycline stock solution that was used in the Vertical Diffusion Cell was prepared with a concentration of 1mg/ml. Such that, tetracycline powder was diluted with methanol to the required amount of concentration, which in this case 1mg/ml Firstly, 10mg of tetracycline was weighed using analytical balance. Then, 10mg of tetracycline was diluted with 10ml of methanol to yield tetracycline concentration of 1mg/ml. The tetracycline solution was placed in Sonicator for 30 minutes until tetracycline powder dissolve completely and mixed well with methanol. The tetracycline stock solution was prepared freshly before use on the same day when the experiment was started.

Preparation of Phosphate Buffer Solution with pH 7.4

Firstly, phosphate buffer solution was prepared by preparing 200ml of acid solution with 800ml of base solution separately before both of the solutions were mixed together. Firstly, 200ml of acid solution was prepared by mixing 0.9073g of potassium dihydrogen phosphate, KH2PO4 with 1.1688g of sodium chloride, NaCl. The mixture of KH2PO4 and NaCl were then topped up with water up to 200ml. Next, 800ml of base solution was prepared by mixing 9.55504g of disodium phosphate, Na2HPO4 and 4.6752g of sodium chloride, NaCl. The mixture of Na2HPO4 and NaCl were then topped up with water until the mixture reached total volume of 800ml. As such, 200ml of acid and 800ml of base solution were prepared respectively. Both of the solutions were then mixed together to yield 1L of phosphate buffer solution. Then, the pH of phosphate buffer solution was adjusted by adding hydrochloride

acid, HCl drop by drop until the pH becomes 7.4. The changes of pH solution while added HCl was monitored by using pH meter.

Preparation of Vertical Diffusion Cell Setup

The circular piece of pig's ear skin was sandwiched securely between the two halves of the Vertical Diffusion Cells with the stratum corneum side facing the donor chamber. The receiver chamber was filled with 5 ml of phosphate buffer solution with pH of 7.4 was continuously stirred and thermo-stated at 32°C throughout the experiment. One ml of the tetracycline stock solution with concentration of 1mg/ml was put in the donor chamber. Four sets of Vertical Diffusion Cell were used for plugged and non-plugged pig's ear skin respectively. Each of the sets was placed with tetracycline stock solution on the donor chamber. Next, the donor chamber will be covered with paraffin film.

Samples Collection

The samples with a volume of 1ml were withdrawn from each sampling ports every 30 minutes for 24 hours by using micropipette. Then, the samples were kept in Eppendorf tube and were kept in the refrigerator (2 – 4°C). However, no samples were taken at time 8 to 18 hours (10 hours gap) where the sets were left running with no samples taken during that time. After each sampling in every 30 minutes intervals, an equal volume of 1ml of phosphate buffer solution was replaced in the sampling port to maintain sink conditions.

Preparation of Standard Curve of Tetracycline with Known Concentrations

Tetracycline stock solutions with known concentrations were prepared to obtain standard curve of absorbance against concentrations (µg/ml) as to compare the absorbance of unknown concentrations of every samples taken from Vertical Diffusion Cell. Six serial dilutions of tetracycline were done by diluting tetracycline stock solution to obtain concentrations of 100µg/ml, 110µg/ml, 120µg/ml, 130µg/ml, 140µg/ml and 150µg/ml respectively. Firstly, Tetracycline stock solution with concentration of 1mg/ml was prepared by diluting 10mg of tetracycline raw powder with 10ml of methanol solution. Then, serial dilutions was done by diluting 1ml of Tetracycline stock solution up to 10ml with phosphate buffer solution (pH 7.4) to obtain working standard solution of 100µg/ml. The steps were repeated again by diluting 1.1ml, 1.2ml, 1.3ml, 1.4ml and 1.5ml of Tetracycline stock solutions to yield working standard solutions of 110µg/ml, 120µg/ml, 130µg/ml, 140µg/ml and 150µg/ml respectively.

Analyses of Samples by Using UV Spectrophotometer

The samples taken from Vertical Diffusion Cell for plugged and non-plugged pig's ear skin were then analysed by using UV Spectrophotometer. The blank that was chosen for the setup of UV Spectrophotometer was distilled water. The samples were analysed with wavelength of 269nm. Then, the reading of each samples were recorded and the concentration of each samples was obtained by comparing the reading of wavelength from each samples with the standard curve of tetracycline with known concentrations plotted earlier.

RESULTS AND DISSCUSSION

Standard curve of Tetracycline

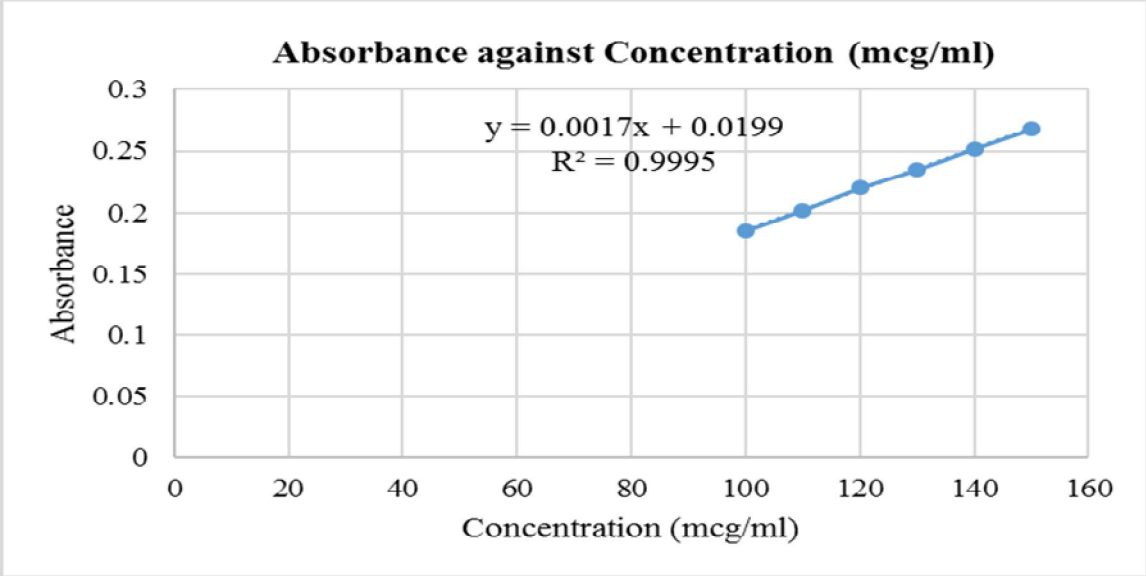

Figure 4: Standard curve of tetracycline

The equation of y = mx + c was obtained from this graph of Figure 4 where y is absorbance value of tetracycline stock solution, m is the gradient of the graph, x is the concentration of tetracycline stock solution with c is the y-intercept of the graph. Such that, from the graph the value of m = 0.0017 with c = 0.0199.

Concentration of Tetracycline through Non-plugged Pig's Ear Skin

The concentration of the respective samples withdrawn from the sampling ports for non-plugged pig's ear skin for each time intervals (every 30 minutes) was calculated based on the equation of y = 0.0017x + 0.0199 that was obtained from the standard curve of tetracycline stock solution where; m = 0.0017 with c = 0.0199. The graph of the concentration of tetracycline for non-plugged pig's ear skin was illustrated in Figure 5.

Table 1: The absorbance value with concentration (µg/ml) of tetracycline permeated through non-plugged pig's ear skin for every 30 minutes time intervals over 24 hours

Time (h)	Absorbance	Concentration (µg/ml)
0.0	0.000	0.00
0.5	0.345	191.24
1.0	0.356	197.71
1.5	0.355	197.12
2.0	0.339	187.71
2.5	0.334	184.77
3.0	0.343	190.06
3.5	0.332	183.59
4.0	0.333	184.18
4.5	0.348	193.00
5.0	0.333	184.18
5.5	0.328	181.24
6.0	0.331	183.00
6.5	0.320	176.53
7.0	0.324	178.88
7.5	0.324	178.88
8.0	0.334	184.76
18.0	0.604	343.59
18.5	0.570	323.59
19.0	0.526	297.71
19.5	0.488	275.35
20.0	0.463	260.65
20.5	0.416	233.00
21.0	0.398	222.41
21.5	0.357	198.29
22.0	0.353	195.94
22.5	0.337	186.53
23.0	0.335	185.35
23.5	0.316	174.18
24.0	0.299	164.18

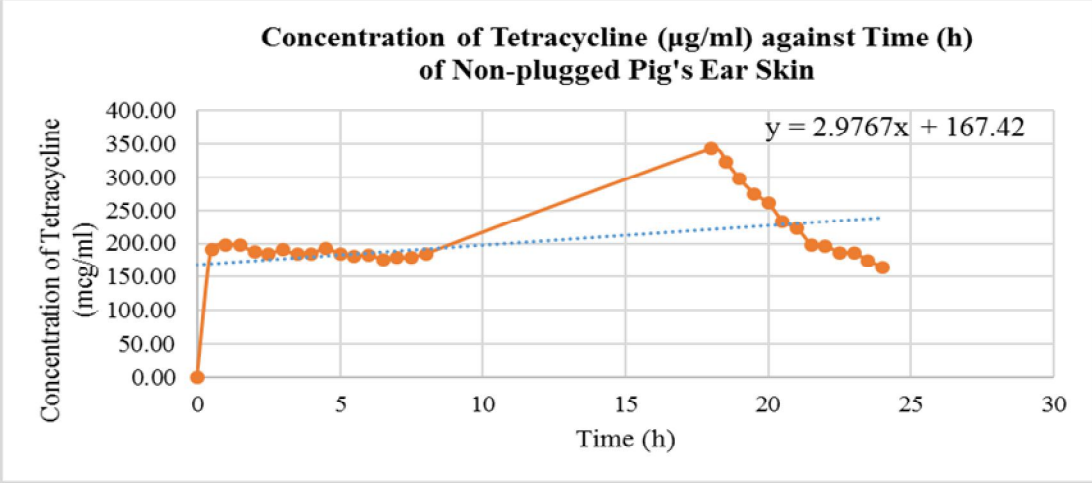

Figure 5 Concentration of tetracycline permeated through non-plugged pig's ear skin

Concentration of Tetracycline through Plugged Pig's Ear Skin

Meanwhile, the concentration of the respective samples withdrawn from the sampling ports for plugged pig's ear skin for each time intervals (every 30 minutes) was calculated based on the equation of y = 0.0017x + 0.0199 that was obtained from the standard curve of tetracycline stock solution where; m= 0.0017 with c = 0.0199. The graph of the concentration of tetracycline for plugged pig's ear skin was illustrated in Figure 6.

Table 2: The absorbance value with concentration (µg/ml) of tetracycline permeated through plugged pig's ear skin for every 30 minutes time intervals over 24 hours

Time (h)	Absorbance	Concentration (µg/ml)
0.0	0.000	0.00
0.5	0.218	112.41
1.0	0.227	116.53
1.5	0.222	121.82
2.0	0.229	118.88
2.5	0.229	123.00
3.0	0.228	123.00
3.5	0.237	122.41
4.0	0.245	127.71
4.5	0.244	132.41
5.0	0.24	131.82
5.5	0.239	129.47
6.0	0.221	128.88
6.5	0.220	118.29
7.0	0.234	117.71
7.5	0.241	125.94
8.0	0.585	130.06
18.0	0.520	332.41
18.5	0.485	294.18
19.0	0.477	273.59
19.5	0.423	268.88
20.0	0.411	237.12
20.5	0.385	230.06
21.0	0.347	214.77
21.5	0.324	192.41
22.0	0.268	178.88
22.5	0.256	145.94
23.0	0.234	138.88
23.5	0.222	125.94
24.0	0.211	118.88

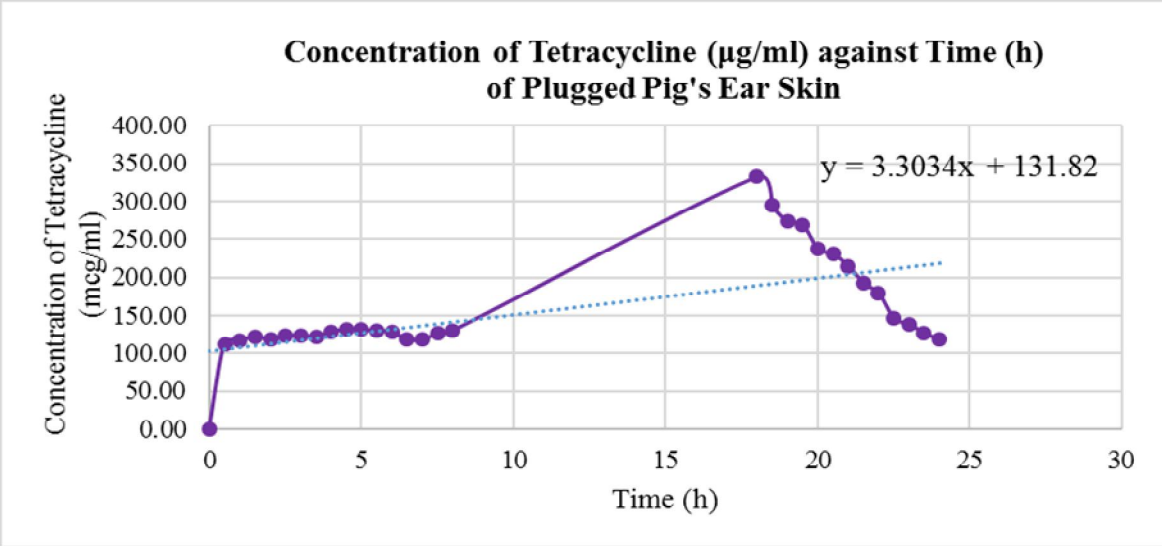

Figure 6: Concentration of tetracycline permeated through plugged pig's ear skin

Comparison of Cumulative of Permeation Drug/Available Permeation Area (µg/cm^2) through Non-plugged and Plugged Pig's Ear Skin

Table 3: Cumulative amount of permeation drug/available permeation area (µg/cm^2) between non-plugged and plugged pig's ear skin

Non-plugged Pig's Ear Skin	Plugged Pig's Ear Skin
0.00	0.00
540.21	355.65
666.53	400.43
776.57	450.10
861.35	475.21
959.09	521.82
1078.43	556.57
1167.53	589.45
1272.91	640.78
1401.89	691.75
1486.01	727.29
1581.75	757.08
1689.13	791.79
1774.24	794.70
1880.62	826.26
1981.69	885.56
2099.34	934.17
2652.40	1611.12
2893.41	1620.46
2899.73	1602.01
3004.78	1664.41
3118.80	1639.87
3187.96	1684.52

3289.69	1701.12
3347.22	1691.06
3452.60	1702.61
3536.72	1648.92
3638.78	1667.82
3711.92	1666.10
3782.08	1679.35

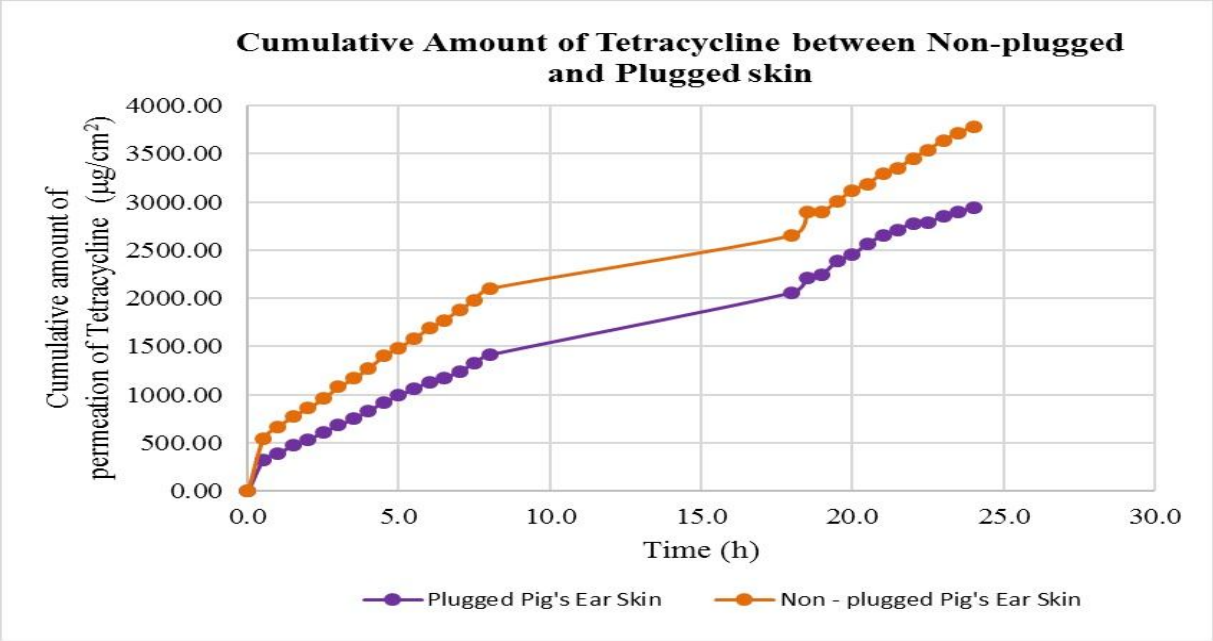

Figure 7: Cumulative amounts of permeation drug/available permeation area (µg/cm^2) between non-plugged and plugged pig's ear skin. The results are statistically significant (P<0.05) between concentration of non-plugged and plugged pig's ear skin.

Calculation of Skin Permeation Parameters

P = Apparent Permeability Coefficient

$$\text{P of plugged skin} = \frac{2.9767}{1000\mu g/ml}$$

$$\text{P of non-plugged} = \frac{3.3034}{1000\mu g/ml}$$

Skin Permeation-Decreasing Ratio (%)

$$= \frac{(\text{P of non-plugged skin}) - (\text{P of plugged skin})}{\text{P in non-plugged}} \times 100$$

$$= \frac{0.0033034 - 0.0029767}{0.0033034} \times 100$$

= 9.889 %

DISCUSSIONS

The present study showed the permeation pathway of tetracycline through non-plugged and plugged pig's ear skin. A significantly higher concentration of tetracycline was observed in non-plugged pig's ear skin in Figure 7 where the hair follicles remain open such that no follicular hair blocking methods were performed on non-plugged pig's ear skin. In contrast, concentration of tetracycline permeated through plugged pig's ear skin were generally lower than the concentration of tetracycline permeated through non-plugged pig's ear skin such that the hair follicles of plugged pig's ear skin has been previously blocked by using silicone grease through hair follicular blocking method. As such, by blocking the hair follicles of plugged pig's ear skin, tetracycline should only pass through the intercellular and intracellular pathways of the skin.

Figure 5 and 6 show the graph of concentration of tetracycline permeated through non-plugged and plugged pig's ear skin respectively. The concentration of tetracycline was found higher at 18 hours as the samples were left for 10 hours gap (no samples were taken during the gap) before next samples collection for both non-plugged and plugged pig's ear skin. However, the concentrations of tetracycline in both non-plugged and plugged pig's ear skin were gradually decreased after that until towards the end of sampling period at 24 hours. The present study is only limited to 24 hours sampling periods where the concentration of tetracycline in both non-plugged and plugged pig's ear skin was still detected at the end of the study. As such, it is recommended for the period of the study to be extending from 24 hours to 72 hours period in further studies as to observe the activity of tetracycline in extended period of time. In preceding study done by Blume-Peytavi *et al.*, 2010, the study of follicular and percutaneous penetration pathways of topically applied minoxidil foam was done for duration of 72 hours. From the study, no minoxidil concentrations were detected 72 hours after application.

Meanwhile, Figure 7 illustrates the cumulative amount of permeation of tetracycline for non-plugged and plugged pig's ear skin where non-plugged pig's ear skin shows higher cumulative amount of tetracycline that was permeated through the pig's ear skin compared to plugged pig's ear skin. The p-value of non-plugged and plugged pig's ear skin was obtained from t-test where the difference is considered to be statistically significant ($P<0.05$) between concentration of non-plugged and plugged pig's ear skin.

These findings on the difference of drug permeated between non-plugged and plugged skin are similar to those of study done by Otberg *et al.,* 2007, where topical application of caffeine carried out on six healthy Caucasian male volunteers show significantly faster absorption of caffeine when the hair follicle orifices were open. With the follicular orifices blocked, caffeine penetration of the skin took much longer time and the maximum measured caffeine blood concentration was generally lower. They concluded that hair follicles may allow a fast delivery of topically applied substances.

A similar finding is also observed on the study done by Blume-Peytavi *et al.,* 2010 on the analysis of penetration pathways of minoxidil foam in six volunteers. The blood levels of minoxidil of volunteers were detected 5 minutes after topical application of the minoxidil foam when follicles were left open. In contrast, minoxidil could not be detected in the blood until 30 minutes after topical application in the setting of blocked hair follicles. As such, the minoxidil absorption took at least twelve times longer in closed follicles. It was demonstrate that hair follicles contribute to the penetration of minoxidil into the blood circulation and support their importance in drug delivery.

Based on the calculation of skin permeation ratio, the value of skin permeation decreasing ratio from non-plugged and plugged pig's ear skin is obtained with a value of 9.89%. As such, nearly 10% difference of concentration of tetracycline drug is permeated between non-plugged and plugged pig's ear skin. From the present study, the difference in permeation decreasing ratio of tetracycline shows that tetracycline permeation route is not largely influence by hair follicles pathway.

However, the limitation of the study was the analysis of sample is performed by using UV spectrophotometer. Thus, further study is needed to analyze the sample by using higher sensitive method of analysis to confirm the permeation pathway of tetracycline is through hair follicles. In this case, new surface ionization mass spectrometry (SI/MS) technique is recommended to be used as an assay method for further studies. This method of analysis was employed in several studies to determine the penetration of drug across skin (Blume-Peytavi *et al.,* 2010; Otberg *et al.,* 2007). This measuring system is based on highly selective detection of minute amounts of drug which enabled a clear distinction to be made between interfollicular and follicular penetration of a topically applied substance Otberg *et al.,* 2007.

Tetracycline should be placed under consideration of pharmaceutical development area to be formulated as drug with nanoparticles size for topically applied drug. In other words, tetracycline should be considered as one of potential drugs if further researches of tetracycline by using sensitive analysis method prove that the difference of tetracycline concentration permeated between non-plugged and plugged skin show considerably higher difference in terms of skin permeation ratio than the present study which only obtained 10% difference. Nanoparticles system is an increasingly implemented strategy in drug targeting and delivery as nanoparticles accumulated preferentially in the follicular openings and the follicular localization was favoured by the smaller particles size (Alvarez-Roman *et al.*, 2004). These finding is beneficial for later development of tetracycline in nanoparticles size for optimization of drug targeting therapy especially for acne treatment.

CONCLUSION

The present findings clearly suggest that the permeation pathway of tetracycline drug into pig's ear skin is through hair follicles. This theory is proven with the evidence of higher concentration of tetracycline was permeated through non-plugged pig's ear skin compared to the concentration of tetracycline permeated through plugged pig's ear skin. The difference of concentration of tetracycline between non-plugged and plugged pig's ear skin is considered to be statistically significant as ($P<0.05$).

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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