Chapter 7

Correlation between Porcine and Human Skin Models by Optical Methods

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Abstract

Background: Topical photodynamic therapy (PDT) using 5-aminolevulinic acid (ALA) and methyl aminolevulinate (MAL) as precursors of protoporphyrin IX (PPIX) have been used in skin cancer treatment and other skin diseases. To establish new topical PDT, protocols are necessary first to conduct studies *in vivo* using animal skin models. The goal of this study is to evaluate the robust correlation between porcine and human skin models *in vivo* by optical methods to confirm the suitability of porcine skin models to predict drug behavior in the human skin on topical PDT protocols. Methods: The study was performed *in vivo* using porcine and human skin models. In human skin, ALA and MAL cream mixture samples were applied to the inner arm in a circular area of 1 cm^2. In porcine skin, the cream was applied on the back in an area of 4 cm^2, over which an occlusive dressing was placed. PPIX production was monitored for up to 5 h using widefield fluorescence imaging and fluorescence spectroscopy techniques. Results: Human skin models showed similar behavior to porcine skin models, which indicates high similarity between both models and confirms that porcine skin is an adequate model to establish new clinical PDT protocols in human volunteers.

Keywords: 5-ALA, MAL, porcine skin, human skin, widefield fluorescence imaging, fluorescence spectroscopy, photodynamic therapy, skin models

1. Introduction

Photodynamic therapy (PDT) constitutes an alternative therapy in the treatment of cancer and skin diseases. The photodynamic reaction comprises the interaction of a photosensitizer (PS), light (lasers, lamps, and LEDs), and oxygen present in the tissue. The photochemical process occurs when the PS absorbs light in one specific wavelength, interacts with subtracts and oxygen, and produces reactive oxygen species (ROS) and singlet oxygen ($^1O_2$), which are the main causes of PDT damage [1, 2]. Topical PDT using topical medication such as 5-aminolevulinic acid (ALA) and its methyl ester (methyl aminolevulinate [MAL]), has been widely employed to treat skin cancer, skin diseases, and aging skin [2–4]. When methyl, ethers, and other groups are added to ALA, its derivatives become more lipophilic, thereby increasing permeability through the skin [5].

ALA and MAL act as precursors of protoporphyrin IX (PPIX), an endogenous PS produced by mitochondria on cells [2]. While ALA and MAL application on PDT has the advantage of being localized and nonsystemic (transdermal application), it has some limitations as regards penetration through the skin [2, 6, 7].

ALA is a hydrophilic compound, making it difficult to cross the biological barriers of the skin, such as cell membranes. However, it has high efficiency in the production of PPIX. On the other hand, MAL has a lipophilic character allowing it to be transported by nonpolar amino acids via passive diffusion (does not require a driver) facilitating the ability to move across biological barriers reaching higher penetration in the desired tissue, and at a lesser cost than the production of PPIX [8, 9].

It is known that PPIX formation by ALA and MAL application in carcinomas is different to PPIX formation in healthy skin, thus there are few studies comparing ALA and MAL in healthy human skin. Lesar et al. compared the production efficiency of PPIX by the application of ALA and its precursors in various parts of the human body [10]. However, in our study we compared ALA, MAL, and mixtures from both on porcine and human skin models. ALA and MAL as precursors of PPIX were chosen in our study since they are used most in clinical topic PDT [11]. Many types of animal models have been suggested to replace human skin in research on transdermal permeation of molecules [8, 12], including primate, porcine, mouse, rat, guinea porcine, and snake models. Nowadays, the use of primates in research is highly constrained [8]. On the other hand, similarities between porcine skin and human skin models have been discussed in many papers [8, 13].

Animal skin differs morphologically from that of human skin with respect to epidermis and dermis thickness, hair follicles, and other characteristics. Despite their many similarities, porcine and human skin differ regarding structure, immunohistochemistry, and function. Notwithstanding, porcine appears the most suitable animal type to replace human skin in test models [14]. Indeed, porcine constitutes the nonrodent species of choice in the preclinical toxicological testing of pharmaceuticals [13].

The prospect of decreasing the number of human volunteers in studies using *in vitro* and *in vivo* methodologies is an advantage in the development of drugs at pharmaceutical companies

[8, 10]. *In vitro* studies using porcine ear skin as a model for human skin have produced positive results, suggesting a high similarity between both skin models [15].

Research has shown that skin barriers vary among species as regards the amount of free fatty acids and triglycerides and density of hair follicles [8]. Stratum corneum (SC) lipid composition (ceramides, free fatty acids, cholesterol, and cholesterol esters) and organization in biological membranes differ from one species to another. According to Godin, the lack of correlation in transdermal drug permeation among species or different application sites in the same animal model is mainly due to variations in skin thickness, the composition of intercellular SC lipids, and a number of skin shafts [8]. Bearing this in mind, research has shown that porcine ear skin is anatomically similar to human skin regarding lipid composition, which confirms its suitability for use as a new animal model to study adnexal glands. In addition, its anatomic and physiologic characteristics with respect to cardiovascular, urinary, integumentary, and digestive systems are similar to those of human skin [13, 16].

Many authors claim that porcine skin models constitute the most relevant animal model for human skin because porcine skin and human skin have similar histological and biochemical properties [8, 9, 15]. Porcine skin is structurally similar to human skin regarding epidermal thickness and dermal–epidermal thickness ratio; their dermis thickness is approximately 3 mm and their SC and epidermis thicknesses are in the region of 21–26 and 70 μm, respectively [8, 13]. The collagen fiber arrangement in the dermis and the SC proteins (glycosphingolipids and ceramides) present in the porcine skin are also similar to those of human skin [8].

While the vascular anatomy of human skin is superior to that of porcine skin, neonatal porcine skin has the same structure, including sweat glands and hair follicles (730 follicles/cm^2), as opposed to 10 follicles/cm^2 found in adult porcine skin [8, 17]. In this way, in this work we performed the tests in animals of 3–4 months of age.

Nowadays, many scientists consider porcine skin a suitable and readily available model for the human skin barrier and often employ it to test topical and transdermal pharmaceutical formulations both *in vivo* and *in vitro*. Indeed, its application in *in vitro* testing is increasing rapidly. Many studies using porcine skin models have compared its permeability with that of human skin and the results show high similarity [18].

Although several studies indicate similarities between porcine skin and human skin models, predictions about drug behavior in human skin based on results from tests using animal models are still under debate. Some authors believe that animal models constitute useful tools in biomedical research, but remark that effects obtained with animal models are not readily transferable to clinical settings [19].

The purpose of this work is to verify whether there is a robust correlation between porcine and human skin models and, if so, confirm that the porcine skin model is the best alternative to prediction studies with human skin volunteers using optical techniques.

In the previous study [19], porcine skin was studied, and in this chapter we can evaluate the correlation between both models. Seven different samples (ALA, MAL, and mixtures from both) were applied to human and porcine skin and their PPIX production was monitored using widefield fluorescence imaging and fluorescence spectroscopy techniques.

2. Materials and methods

2.1. Chemicals

The PPIX precursors used in this study were ALA and MAL (final concentration of 20%), which were dissolved in different proportions in an oil-in-water (O/W) emulsion. Seven samples (ALA, MAL, and mixtures from both) were prepared in the following proportions: M1 (100% ALA), M2 (80% ALA and 20% MAL), M3 (60% ALA and 40% MAL), M4 (50% ALA and 50% MAL), M5 (40% ALA and 60% MAL), M6 (20% ALA and 80% MAL), and M7 (100% MAL). The emulsion or cream preparation was previously described [19].

Commercial ALA and MAL were obtained from PDT-PHARMA (Cravinhos, São Paulo, Brazil) and were prepared immediately prior to use without previous solubilization because the drug presents elevated solubility in the base cream used.

2.2. Human study approval

This study used a protocol along the lines of the procedures established by Brazil's Human Research Ethics Committee (no. 13556713.8.0000.5504). In addition, a written informed consent was obtained from all participants. Ten female patients aged around 25 years with the clinical diagnosis of normal skin were recruited for this study. To be considered eligible, a patient had to be free of skin disorders on both arms. The volunteers were all women to decrease the variable numbers in the study.

All patients had either Fitzpatrick skin type II (50%) or III (50%). Patients with lesions on the target area or with porphyrin were excluded. Additional exclusion criteria included male volunteers, pregnant or lactating female volunteers, those allergic to ALA and MAL, and volunteers less than 20 and more than 35 years of age. The volunteer protocol followed the standardization previously described [2].

2.3. Topical application of the skin models

The study was performed *in vivo* in human and porcine skin models using ALA, MAL, and mixtures from both cream applications (samples: ALA, M2, M3, M4, M5, M6, and MAL). The cream was applied with a sterile spatula, with a density of 32 mg/cm^2, in tests involving both animals and human volunteers.

In human skin, the cream was applied in seven areas (a circular area of 1 cm^2 each) on each volunteer's right or left inner arm (10 woman human volunteers). Before cream application, the area was cleaned with serum. Skin autofluorescence was used as the control for each cream sample (ALA, M2, M3, M4, M5, M6, and MAL). Data were compared for each treatment group for the same volunteer and among volunteers.

In porcine skin, the cream was applied in a square area of 4 cm^2 (applied on the back). To prepare the cream application it was necessary to remove the hairs from the back. Animal testing involving porcine skin models was performed as described above with seven cream samples (ALA, M2, M3, M4, M5, M6, and MAL) in triplicate (in regard to conditions as well as experiments) [19].

The areas were dressed with an occlusive mask to protect them from light. The cream was removed prior to autofluorescence collection at the skin by fluorescence spectroscopy and widefield fluorescence imaging. After collection, the same amount of cream was applied again under the same conditions at every full hour during 5 h. The experimental procedure was previously described (**Figure 1**) [2, 19].

2.4. Fluorescence collection: widefield fluorescence imaging and fluorescence spectroscopy

A widefield fluorescence imaging system was used for image acquisition. It is a commercial device, produced by MM Optics, Sao Carlos, Brazil, called EVINCE. In brief, the widefield imaging system consists of a lighting device based on LEDs, emitting around 405 nm coupled to a digital camera for image acquisition [2, 19, 20]. The measurements were taken for each sample and different times of PPIX formation. The images obtained by widefield fluorescence were assessed quantitatively using a routine written in Matlab program. The program has defined an array separating the red, green, and blue (RGB) channel colors.

For pixel count analysis, standardization was obtained dividing the red channel by the green channel. This was done to avoid the effects of different shutter speeds and ISO settings for image acquisition. Finally, fluorescence (pixel count) was determined by summing the values of all red channels divided by the sum of all values of the green channel. With these data, it was possible to quantify PPIX production (which shows red fluorescence) in amount and uniformity on the skin extension [2, 19].

For fluorescence spectroscopy analysis, a system with two excitation lasers was used, emitting at 408 nm and 532 nm, respectively. The investigation fiber probe in the Y shape directs the excitation laser to the tissue, while the second arm of the Y shape collects the re-emitted light from the tissue and delivers light to a spectrophotometer. The spectrophotometer used was the USB2000 (Ocean Optics®, USA). A filter was used to remove backscattered light so that only the fluorescence was collected [2, 19, 21].

We recorded the fluorescence spectrum in contact with the tissue at five points in the area where the cream was applied, and equivalent autofluorescence collection was performed. The evaluations from spectral analysis were normalized by total area and subtracted from

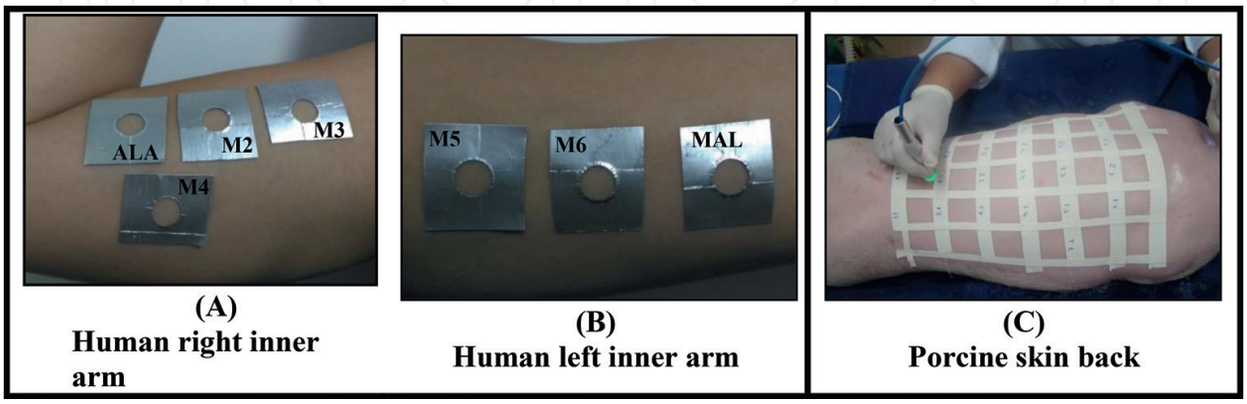

Figure 1. Application of different cream samples (ALA, M2, M3, M4, M5, M6, and MAL) at the skin: (A) human right inner arm; (B) human left inner arm; and (C) porcine skin back.

the autofluorescence. The spectrum evaluations were performed using an Origin 9 program as previously described in our publication [2, 19]. A spectrometer and widefield fluorescence imaging equipment collected skin fluorescence at every full hour during 5 h. At the end of the fluorescence analysis, the cream mixtures were reapplied to the treatment area and covered with an occlusive dressing.

3. Results

Figure 2 shows the PPIX formation after 3 h of cream incubation for human and porcine skin obtained by widefield fluorescence imaging. **Figure 3** shows the quantitative analysis of the PPIX formation by counting pixels of the images and spectroscopy collection acquired by fluorescence techniques. The result of this analysis was acquired by means of the average data collected from volunteers and animals. The results of the porcine skin model were also published previously [19]. The results in **Figure 3** show that the high amount of PPIX production in human and porcine skin occurs for all cream samples (ALA, M2, M3, M4, M5, M6, and MAL) after 3 h of cream application.

Fluorescence imaging shows that PPIX production is heterogeneous for healthy skin in both skin models. Even so, it is possible to verify the differences in PPIX formation to ALA, MAL, and mixtures from both. The results suggest that PPIX formation is greater for ALA than for MAL for both models. In addition, PPIX formation of all sample cream mixtures from ALA and MAL (M2, M3, M4, M5, and M6) was more elevated than MAL and is similar to ALA.

In addition, it is important to mention that for porcine skin preparation it was necessary to shave the back, and for human skin preparation the area was cleaned with physiological serum. This previous skin preparation can interfere with cream sample penetration on the skin as well as PPIX production. This explains the lowest PPIX production for all samples in human skin models when compared to porcine skin models (**Figure 3**).

Figure 4 shows the kinetics study on human skin only, wherein the monitoring of PPIX production was carried out during 5 h, and the measurements were carried out hourly for both

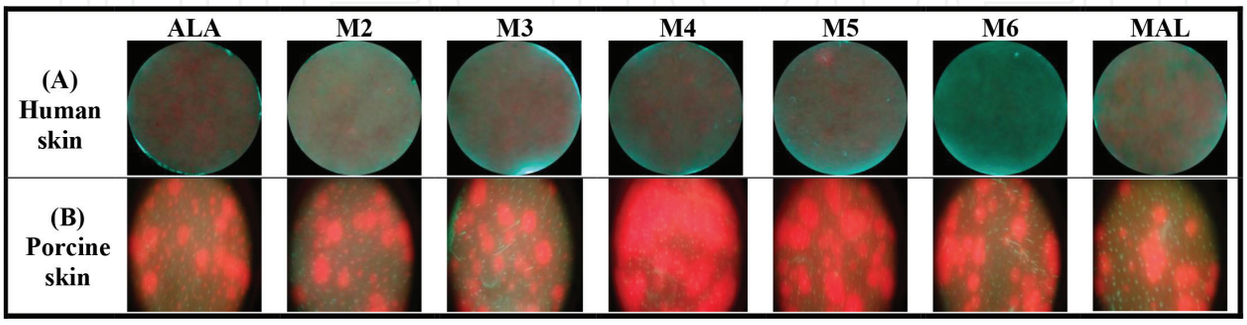

Figure 2. Widefield fluorescence imaging after 3 h of cream sample application (ALA, M2, M3, M4, M5, M6, and MAL): (A) PPIX production in human skin (inner arm) and (B) PPIX production in porcine skin back.

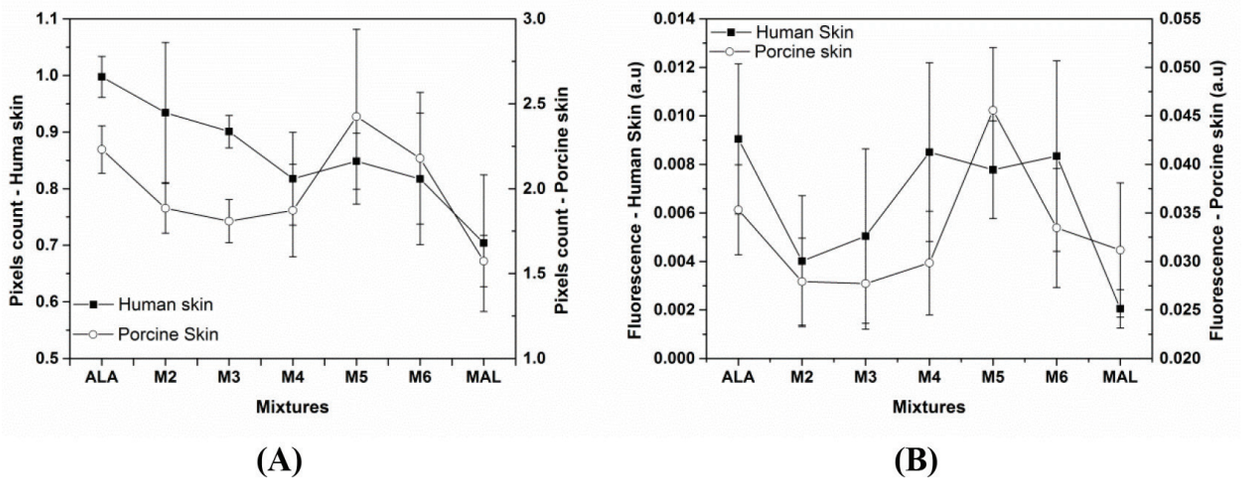

Figure 3. Analysis of PPIX production in human and porcine skin models after 3 h of cream sample application evaluated by (a) widefield fluorescence imaging and (B) spectroscopy fluorescence.

fluorescence techniques. From this study, it was possible to analyze which sample PPIX was produced quickest, and by means of a parameter called the index of fluorescence (IF_{50}) it was possible to quantify the PPIX production in minutes. IF_{50} means 50% of maximum fluorescence value obtained for 5 h.

Although **Figure 4** presents the results of the human skin study, **Table 1** shows the IF_{50} results for the study in human and porcine skin. We have included it here for best comparison. The graphs of the kinetics study in porcine can be observed in previously published work [19].

The results for the kinetics study in the human skin model show that PPIX production is faster with ALA than MAL and cream samples (M3, M4, M5, and M6) for both fluorescence analyses. However, the mixtures M3, M4, and M5 presented better results for PPIX production than MAL in the kinetics study (IF_{50} values). These results suggest that these differences may be due to high variability in human skin experiments. Perhaps these differences can be reduced by using a number of human volunteers.

The results for the kinetics study from **Table 1** show that PPIX production in 5 h (IF_{50}) in human skin models is faster for ALA than MAL, and the opposite occurs for porcine skin models where PPIX production is faster for MAL than ALA. This can be explained by the previous preparation for porcine skin where we can suggest that PPIX production by MAL can be optimized. The other sample creams (M3, M4, M5, and M6) show the same behavior considering the standard deviation.

The values found for IF_{50} through the widefield fluorescence imaging data were closer to human and porcine skin models than the IF_{50} values collected by fluorescence spectroscopy. We believe that this occurred because PPIX production is heterogeneous and the fluorescence spectroscopy measurements are punctual. This punctual fluorescence collection data of PPIX production can suggest false negative or false positive results. On the other hand, by using widefield fluorescence imaging, we can evaluate all PPIX production on the skin surface.

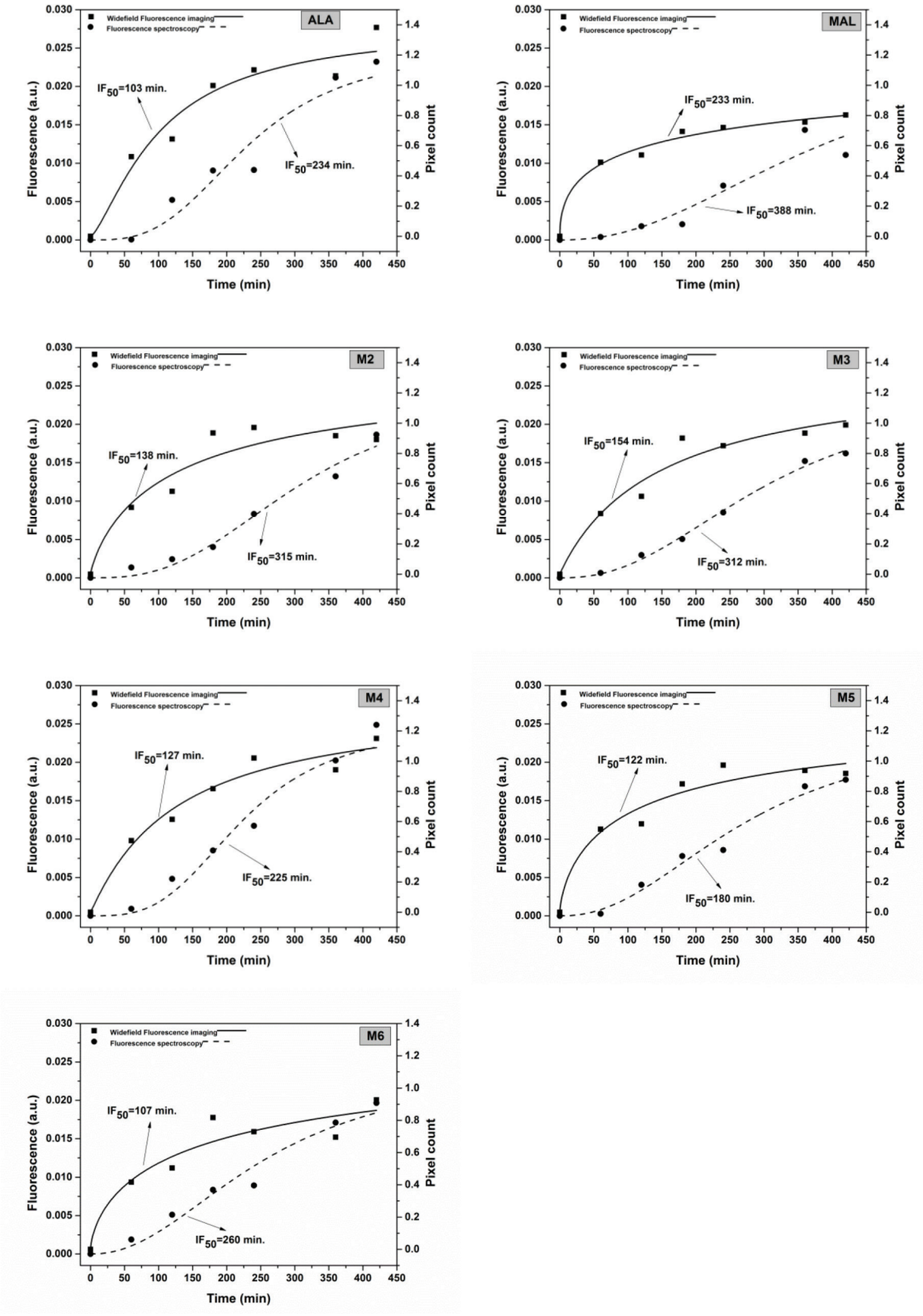

Figure 4. Kinetics of the PPIX production in human skin models by fluorescence spectroscopy and widefield fluorescence imaging evaluations for all cream samples (ALA, M2, M3, M4, M5, M6, and MAL).

Samples	Widefield fluorescence imaging IF_{50} (min)		Fluorescence spectroscopy IF_{50} (min)	
	Human skin	Porcine skin	Human skin	Porcine skin
ALA	103 ± 15	120 ± 10	234 ± 18	230 ± 7
M2	138 ± 27	134 ± 6	315 ± 13	
M3	154 ± 18	40 ± 60	312 ± 6	114 ± 16
M4	127 ± 14	128 ± 8	225 ± 13	17 ± 20
M5	122 ± 21	97 ± 7	280 ± 12	131 ± 17
M6	207 ± 38	120 ± 20	260 ± 15	187 ± 25
MAL	233 ± 18	70 ± 5	388 ± 37	131 ± 9

Table 1. IF_{50} values for widefield fluorescence imaging and fluorescence spectroscopy collected over time (5 h) after sample cream application on human and porcine skin model surfaces.

Figure 5 shows the correlation linear fitting to fluorescence measurements obtained through widefield fluorescence imaging and fluorescence spectroscopy. The fitting in **Figure 5** shows that the red fluorescence signal emitted by PPIX in the porcine and human skin was measured at 3 h following application of ALA and MAL cream mixtures. These results confirm that there is a correlation between both models since the equation line factor obtained was 0.9824, bordering on 1.0, the ideal linear fitting number.

The same linear fitting was performed for fluorescence collected through fluorescence spectroscopy (results not presented here). However, we do not find a correlation between both models (human and porcine skin) by this optical technique due to high variability during fluorescence spectroscopy collection.

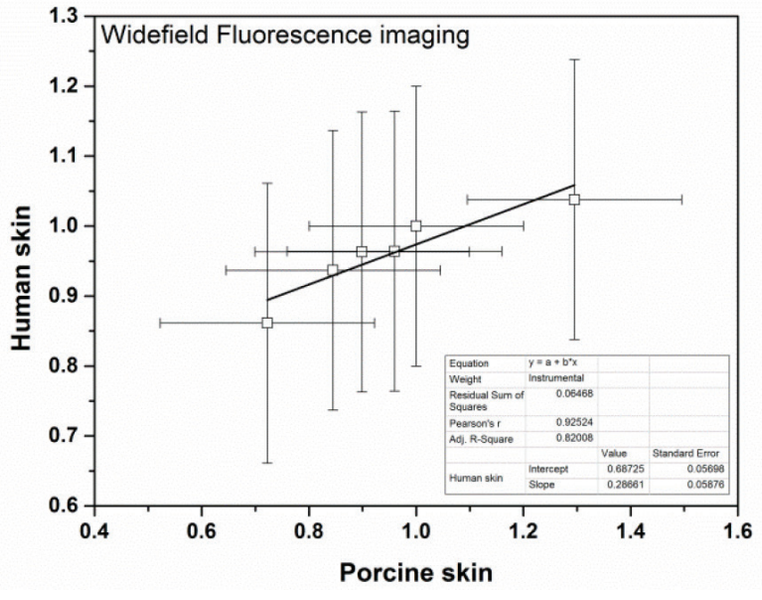

Figure 5. The best correlation analysis between human and porcine skin models by widefield fluorescence imaging.

The fitting results shown in **Figure 5** indicate the best correlation between porcine and human skin models by widefield fluorescence imaging measurements. The possibility of predicting drug behavior on transdermal skin application promotes the success of clinical topical PDT treatment.

4. Discussion

The aim of the study was to evaluate PPIX formation due to ALA, MAL, and cream sample mixtures from both (M2, M3, M4, M5, and M6) application on normal skin models (porcine and human) and then to show that there is a narrow correlation between both models. In this work we use ALA and MAL on topical application as the precursor of PPIX, since these are the most common drugs applied to clinical topical PDT. The fluorescence measurements were collected after 3 h of cream incubation time since this time is also applied to clinical PDT [19, 22].

In our group [22] the clinical PDT studies on skin cancer are done using 20% ALA and MAL cream application in 3 h of incubation time before light irradiation. During this time, PPIX production is elevated since the previous preparation was performed (curettage).

The fluorescence measurements were done using two techniques: fluorescence spectroscopy and widefield fluorescence imaging. With fluorescence spectroscopy using a 532 nm laser (green light) it is possible to evaluate the skin at greater depths (reaching the dermal papillae) when compared with widefield fluorescence imaging using a 405 nm LED (violet light) bringing images from PPIX on the superficial skin [2, 19].

In the study the choice of animal age had great influence; in agreement with the literature the thickness of porcine skin is similar to human skin at around 2 months after birth [8, 13].

PPIX formation on normal skin is not homogeneous and depends on ALA, MAL, and mixtures from both (M3, M4, M5, and M6) penetration through the skin; evaluations using images by widefield fluorescence imaging can be useful and decrease the variability on experiments. Fluorescence spectroscopy evaluation, despite being collected punctually, which can lead to erroneous measurements and high variability, reveals information about PPIX formation on the deeper skin [2, 19] and is important to understand the replacement mechanism of PPIX from deeper layers up to superficial skin layers.

As shown by Valentine et al. [23], there was no difference after increasing the amount of PPIX using ALA and MAL when analyzed by fluorescence spectroscopy using a laser emission at 405 nm (violet light). Fluorescence emission due to 405 nm illumination allows us to measure the output of PPIX on the superficial skin (stratum corneous and superior epidermis). In our work, this superficial skin analysis was performed using widefield fluorescence imaging.

There are few studies concerning the comparison of ALA and MAL in healthy human skin, but Lesar et al. [10] compared the formation efficiency of PPIX from these precursors in various parts of the human body (arm, forearm, back, and legs) with fluorescence (4–29 h) after topical application. They then observed that there were differences in PPIX production, which applied regardless of where the ALA accumulated more PPIX, but the location (back) where they applied the tape striping difference was only after 24 h.

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The kinetics study observed that ALA, M4, and M5 indicated the least time of PPIX production (high PPIX production velocity) at the skin. Both studies, human and porcine skin, showed the same behavior. IF_{50} values acquired by widefield fluorescence imaging for both models were very close, with the exception of M3, M6, and MAL. Thereby, it is possible to appreciate the similarity of porcine skin with human skin by first performing clinical tests on porcine skin.

However, it is known that *in vitro* and *in vivo* experiments using the same species show less variability than experiments using human volunteers. The authors suggest that human experiments are done using a greater number of volunteers. The measurement of the correlation coefficient proved that porcine and human skin models have the same behavior with respect to the production of PPIX in quantity as well as in speed of PPIX production through optical methods. The correlation coefficient is a measure of how well the predicted values from a forecast model fit with the real data. We suggest that the best correlation is between porcine and human skin by widefield fluorescence imaging, suggesting this optical method as an important tool to develop new clinical topical PDT protocols.

5. Conclusion

The correlation found between human and porcine skin models measured by widefield fluorescence imaging confirms that porcine skin can be used for establishing human protocols in clinical topical PDT using ALA, MAL, and mixtures from both. The capacity of porcine skin models to predict PDT results in humans can be beneficial to clinical studies optimizing PDT treatment on patients.

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