Optical properties of mice skin for optical therapy relevant wavelengths: influence of gender and pigmentation

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Abstract

Red and near-infrared light have been widely employed in optical therapies. Skin is the most common optical barrier in non-invasive techniques and in many cases it is the target tissue itself. Consequently, to optimize the outcomes brought by light-based therapies, the optical properties of skin tissue must be very well elucidated. In the present study, we evaluated the dorsal skin optical properties of albino (BALB/c) and pigmented (C57BL/6) mice using the Kubelka-Munk photon transport model. We evaluated samples from male and female young mice of both strains. Analysis was performed for wavelengths at 630, 660, 780, 810 and 905 nm due to their prevalent use in optical therapies, such as low-level light (or laser) and photodynamic therapies. Spectrophotometric measurements of diffuse transmittance and reflectance were performed using a single integrating sphere coupled to a proper spectrophotometer. Statistic analysis was made by *two-way* ANOVA, with Tukey as post-test and Levenne and Shapiro-Wilks as pre-tests. Statistical significance was considered when $p<0.05$. Our results show only a slight transmittance increment (<10 %) as

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wavelengths are increased from 630 to 905 nm, and no statistical significance was observed. Albino male mice present reduced transmittance levels for all wavelengths. The organization and abundance of skin composing tissues significantly influence its scattering optical properties although absorption remains constant. We conclude that factors such as subcutaneous adiposity and connective tissue structure can have statistically significant influence on mice skin optical properties and these factors have relevant variations among different gender and strains.

Keywords: tissue optics, light, dosimetry, phototherapy, photodiagnosis.

1. Introduction

Red and near-infrared radiation have been broadly employed in biomedical optics. Even though some of the light based biomedical technologies are still of exclusive academic use, light has already become a powerful tool in health sciences with many applications in diagnostics and therapeutics.

Skin is the most common optical barrier for non-invasive interventions and, occasionally, it is the tissue of interest. Accurate light parameters for optical therapies in general are the diverging points between unsatisfactory and effective responses. Consequently to pursuit optimum therapeutic results, employing trustable light dosage inside the therapeutic windows, the optical properties of skin must be very well understood (Anderson & Parrish, 1981; Jacques, 2010).

Skin is a highly complex organ and is yet the largest one in the human body. It is composed of multiple layers of many different cell populations and structural proteins. Such layers are subject of wide variations in thickness and composition – even in a single organism – making light-skin interactions rather heterogeneous (Curtis, Calabro, Galarneau, Bigio, & Krucker, 2011). As laboratory mice with

different gender and skin pigmentation are constantly used to assay phototherapies, to elucidate the influence of such aspects drove the motivation of this study.

The photon transport theory proposed by Kubelka & Munk (KM) provides a simple analytical solution to evaluate global absorption (A_{KM}) and scattering (S_{KM}) optical properties of turbid media (Kubelka & Munk, 1931). The phenomenon is described by two differential equations where their solutions can be written in terms of sample's thickness (D), diffuse transmittance (T_d) and reflectance (R_d). The probabilities of light absorption and scattering events are directly proportional to the A_{KM} and S_{KM} coefficients (Anderson & Parrish, 1981; Kubelka & Munk, 1931). In the present study we experimentally evaluated the optical properties of depilated dorsal skin of male and female mice, from pigmented and albino strains, using the KM method.

2. Material and Methods

2.1. Animals and samples

A total of 20 healthy mice, five males and five females from each strain (C57BL/6 and BALB/c) aging from 4 to 6 weeks were used for *ex vivo* skin sampling. Before the experimental period, all animals were individually housed in acrylic plastic isolators in a 12 h light/dark cycle, and fed with granulated food and water *ad libitum*. All animal procedures, care, and handling were carried out according to the ethical principles of animal experimentation formulated by the Brazilian College for Animal Experimentation (COBEA) and were approved by the local Ethics Committee on Animal Research and Care of IPEN/CNEN-SP.

Animals were euthanized by cervical dislocation, and hair excess on the dorsal region was removed using an electric shaver. In order to minimize light interaction with any remaining fur, skin was depilated with a thioglycolate-based chemical (Veet Cream®, Reckitt Benckiser, Brazil). Whole thickness skin slabs of 2 x 2 cm were then excised. To provide mechanical support, each sample was positioned between two microscope slides, under minimal pressure, as presented in figure 1.

2.2. Spectroscopy analysis

Spectroscopic measurements were carried out by a proper spectrophotometer (Cary 5000, Agilent®, Australia) coupled to a single integrating sphere (Internal DRA-2500 (PMT), Agilent®, Australia). The spectra for transmittance and reflectance were measured ranging wavelengths from 630 nm to 905 nm.

The freshly excised dorsal skin samples were placed between two microscopy slides (fig. 1) and the Fresnell reflection provided from the glass slides surfaces were accounted in a baseline calibration. Transmittance measurements were obtained placing the samples in position “a” (fig. 1) and the reflectance spectra were obtained by shifting the sample to position “b”.

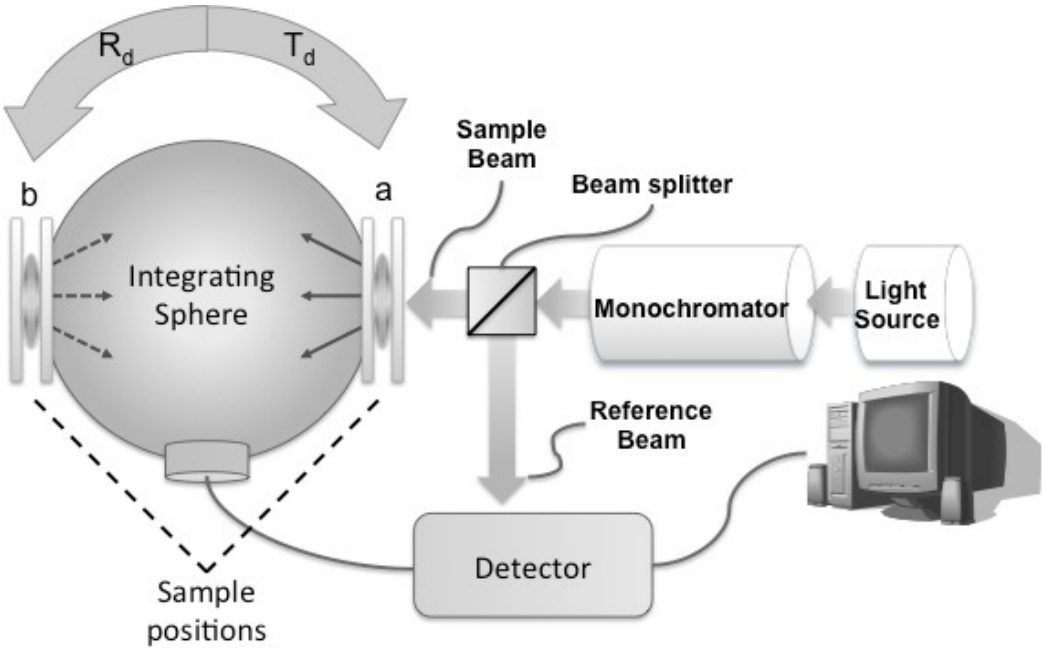

Figure 1. Experimental setup and sample positioning for spectrophotometric measurements. Diffuse transmittance measurements were performed with sample positioned in **a**, and for reflectance sample was positioned in **b**. Sample beam always propagated in the epidermis-dermis sense.

We calculated the optical coefficients A_{KM} and S_{KM} according to the following equations (Anderson & Parrish, 1981; Kubelka & Munk, 1931):

$$A_{KM} = (x-1)S_{KM} \qquad (1)$$

$$S_{KM} = \frac{1}{yD}\ln\left[\frac{1-R_d(x-y)}{T_d}\right] \qquad (2)$$

Where D [cm] is the sample thickness and the parameters *x* and *y* are given by equations 3 and 4:

$$x = \frac{1+R_d^2-T_d^2}{2R_d} \qquad (3)$$

$$y = \sqrt{x^2-1} \qquad (4)$$

2.3. Histological analysis

Following spectroscopic measurements, skin samples were fixed in 10 % buffered formalin and processed for standard hematoxylin and eosin histological staining. Three photomicrographs were obtained (Leica Microsystems, Wetzlar, Germany) from each skin sample and an experienced pathologist with no prior knowledge of the samples analyzed the images. Separate thickness measurements of epithelium, dermis, subcutaneous tissue (adipose tissue and skeletal muscle) were performed in three different areas of each image in a standardized manner (left extremity, middle and right extremity) using the ImageJ software (NIH, USA) for quantifications. A descriptive analysis was also performed.

2.4. Statistical analysis

Samples were found to have normal distribution (Shapiro–Wilks test) and homogeneous variance (Levene's test), thereby we applied the *two-way* ANOVA test for multiple comparisons and used Tukey as post-test. Statistical significance was considered if $p<0.05$.

3. Results

Representative photomicrographs of the samples are presented in figure 2, and quantified mean values of tissues thickness of each group are presented in table 1. Briefly, we observed a greater abundance of adipose tissue in females with significant reduction in the thickness of the dermis when compared to males (table 1 and fig. 2).

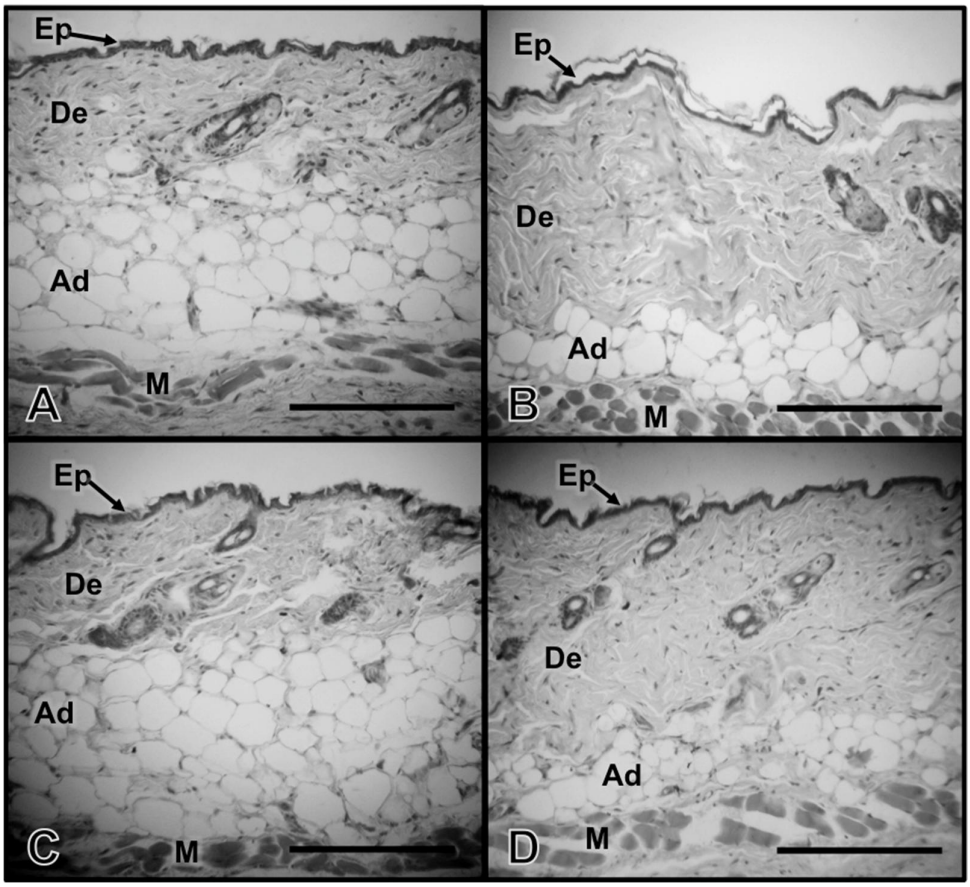

Figure 2. Representative photomicrographs of female **(A)** and male **(B)** albino (BALB/c) mice, and, female **(C)** and male **(D)** black (C57BL/6) mice. **Ep** corresponds to epidermis, **De** to dermis, **Ad** to adipose tissue and **M** to muscle. Observe the thickness variations of dermis and adipose tissues between genders. Scale bar corresponds to 100 μm and samples were stained by standard hematoxylin and eosin.

Table1. Mean thickness of epidermis, dermis, adipose tissue, muscle layer and subcutaneous connective tissue of mice skin samples.
Errors represent standard deviation of the mean.

	BALB/c		C57BL/6	
	Female	**Male**	**Female**	**Male**
	Tissue thickness (μm)			
Epidermis	6.1	5.1	6.6	6.3
Dermis	81.0	221.7	98.4	128.4
Adipose tissue	122.5	60.1	134.7	86.5
Muscle	45.9	45.3	41.7	45.3
Conective tissue	263.6	416.2	258.8	273.7
TOTAL	**519.0 ± 30.2**	**748.3 ± 32.3**	**540.1 ± 51.7**	**540.7 ± 21.4**

The average optical parameters of the evaluated groups are presented in figure 3. Observe that only male albino mice displayed substantial lower transmittance (fig. 3A) when compared to other groups. Over the spectral region we observed, absorption coefficients show only a slight increase tendency as wavelengths approach to the water absorption peaks in the infrared region (>960 nm). However, light-scattering properties of all groups are inversely proportional to wavelengths, in a decay rate that resembles a Rayleigh-Mie scattering curve (fig. 3C).

Albino males have a significantly thicker skin tissue due to greater volume of connective tissue in the dermis and below the muscular layer (table 1, figure 2). Probably, stronger scattering presented by males in the red region (630-660 nm, fig. 3C) occurs due to greater abundance of fibrous proteins (mainly collagen) in thicker connective tissue layers. Yet, it can be remarked that light transmittance presents greater variation when comparing albino males to other mice than when comparing each group at 630 nm with its correspondent at 905 nm (fig. 3A).

As can be seen in table 1 and figure 2, male and female black mice have in average the same skin thickness; however, tissue layers are different in proportion. Such abundance of connective tissue in males, with anisotropic layers of collagen,

may have contributed to the increased scattering properties of male mice skin for the black strain, and consequently, the albino male mice, regarding their female counterparts. In fact, even purified fibrous proteins, such as collagen and keratin, are highly scattering materials (Millington, 2012). Thus, it can also be corroborated that connective tissues may have stronger scattering properties than adipose tissue due to greater presence of collagen fibers and other fibrous proteins. Additionally, no significant differences were observed among absorption coefficients indicating that all disparities between light transmission levels are mainly - if not exclusively – consequence of variable tissue thickness and scattering properties.

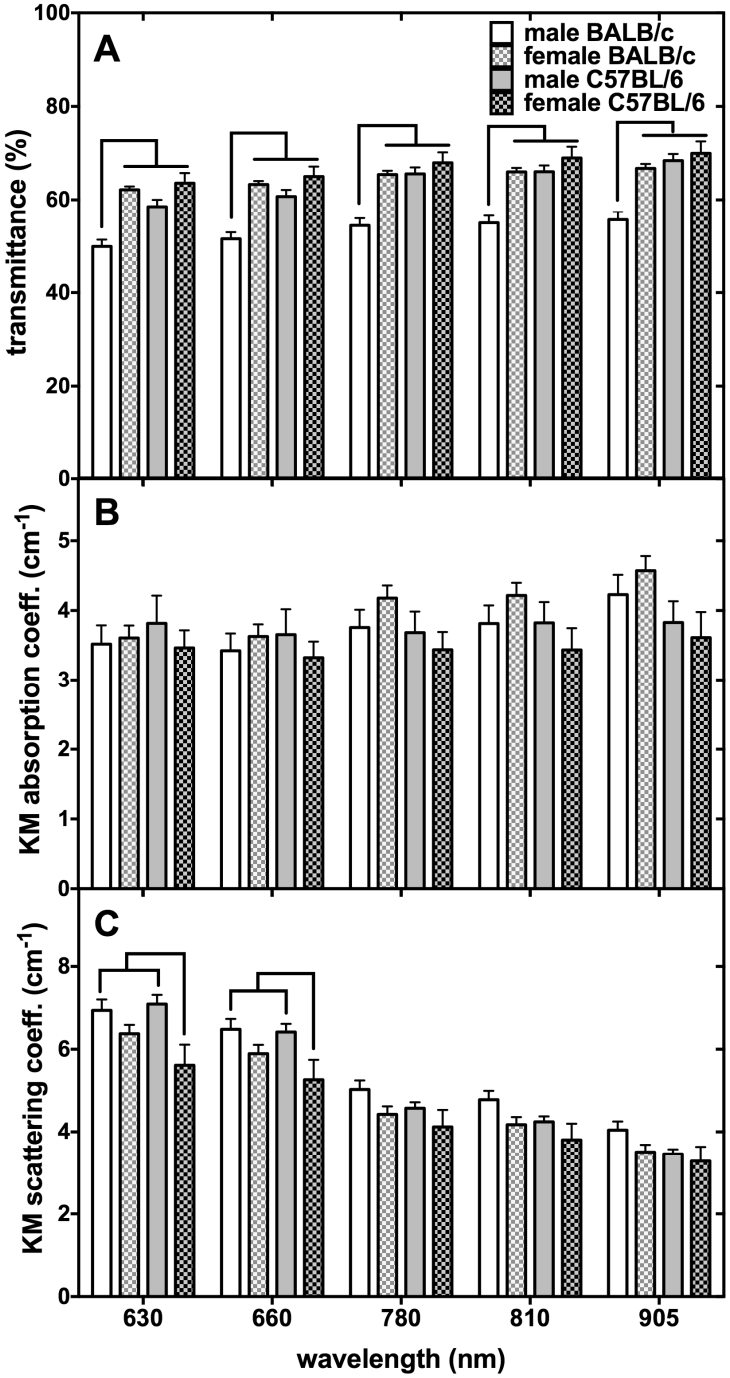

Figure 3. Averages and standard errors relative to diffuse transmittance **(A)** and Kubelka-Munk absorption **(B)** and scattering **(C)** coefficients.

4. Discussion

Since the beginning of the 1980's the optics of human skin has been investigated allowing the development of several optical methods (Anderson & Parrish, 1981). Today, application examples encompass laser Doppler flowmetry, optical coherence tomography, bioluminescence monitoring, confocal and multi-photon microscopies and laser speckle imaging among others. In the context of optical therapies, accurate light parameters are in general the diverging points between unsatisfactory (or null) results and highly effective responses (Jacques, 2010; Chung, Dai, Sharma, Huang, Carroll, & Hamblin, 2012).

Calabro *et al.* identified gender as a significant source of variation in optical reflectance measurements of mice skin *in vivo* and illustrated that variations in the dermal layer thickness and its collagen density are the key explanatory variable (Calabro et al., 2011). These findings are in good agreement with our observations. In occasions of optical measurements where a weak luminescent body is located underneath any tissue this characteristic may lead to low signal detection and relatively more blurred images are obtained decreasing the techniques accuracy. The very same situation can be observed in optical imaging where short wavelength fluorescent probes are employed for observing subcutaneous targets.

Although it is a common assumption that wavelengths in the infrared region are better suited to optimize the transmission of light in biological tissues, our results show only a slight transmittance increment (<10 %) as wavelengths are increased from 630 to 905 nm, and yet no statistical significance was observed. This same level of variance could be observed between experimental groups, on the same wavelength (fig. 3A). Therefore, the light transmittance capability in mice skin depends, in similar magnitude, on the wavelength and composition of tissue present in the experimental

groups. Thus, it can be suggested that light therapy dosimetry targeting subcutaneous tissues deserve better attention at light attenuation due to different skin features rather than what wavelength to use (at the observed spectral range).

This study evaluated the optical properties of mice skin in some wavelengths of technical-scientific interest, only observing the importance of strain and gender. This is an important limitation of this initial study and therefore the development of novel investigations monitoring a broader range of variables is yet necessary to better understand which are the real important parameters to optimize optical applications in biomedical sciences. Also, a comparative study showing the divergences between the optical properties of mice and human skin could be an interesting approach to possibly develop a translational dosimetry system based on tissue optics.

5. Conclusion

We conclude that skin features such as subcutaneous adiposity and connective tissue structure and abundance can have significant variation among genders, directly influencing the mice skin optical properties. Only a slight transmittance increment (<10%), with no statistical significance, is observed as wavelengths are increased from 630 to 905 nm. Connective tissues may have stronger scattering properties than adipose tissue due to greater presence of collagen fibers and other fibrous proteins. Additionally, no significant differences were observed among absorption coefficients indicating that all disparities between light transmission levels are mainly - if not exclusively – consequence of variable tissue thickness and scattering properties.

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