FULL ARTICLE

Sex, but not skin tone affects penetration of red-light (660 nm) through sites susceptible to sports injury in lean live and cadaveric tissues

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Red-light treatment is emerging as a novel therapy for promoting tissue recovery but data on red-light penetration through human tissues are lacking. We aimed to: (1) determine the effect of light irradiance, tissue thickness, skin tone, sex and bone/muscle content on 660 nm light penetration through common sites of sports injuries, and (2) establish if cadaver tissues serve as a useful model for predicting red-light penetration in live tissues. Live and cadaver human tissues were exposed to 660 nm light at locations across the skull, spinal cord and upper and lower limbs. Red-light was produced by a light emitting diode array of various irradiances (15–500 mW/cm²) and measured by a light-probe positioned on the tissue surface opposite to the light emitting diodes. 100 mW/cm² successfully penetrated tissue <50 mm thick; a disproportionate irradiance increase was required to achieve deeper penetration. Penetration was unaffected by skin tone, increased with irradiance and relative bone/muscle composition, and decreased with greater tissue thickness and in males. Live and cadaveric tissue penetration did not differ statistically for tissues <50 mm but cadavers required more red-light to penetrate >50 mm. These results assist clinicians and researchers in determining red-light treatment intensities for penetrating human tissues.

KEYWORDS

LED treatment, photobiomodulation, red light therapy, sports injuries, sprain, strain

1 | INTRODUCTION

While important for overall wellbeing, sports often result in injuries that contribute to an increased burden on the health care system. In the United States, almost half a million young school-aged athletes suffered severe injuries during sports, with approximately two-thirds suffering fractures and/or ligament sprains [1]. The most common sites of

injury are the knee/lower limb and the upper limb [1–3]. Therapies that can effectively treat muscle, bone, tendon, ligament and/or nervous system injuries, including managing inflammation and pain, would be beneficial to athletes at all levels of competition.

The biological effects of low power laser were first reported in 1968 [4] and the use of light emitting diodes (LEDs) for therapeutic applications of photobiomodulation

(PBM) subsequently popularised following the serendipitous finding of improved wound healing by astronauts using LEDs for plant growth experiments in space [5–7]. Since these initial observations, various studies have emerged investigating the effect of PBM on injury, including pain modulation and wound healing in a vast variety of in vitro and in vivo experiments.

Of interest to sports medicine are observations of improved collagen deposition in skeletal muscle [8], reduced oedema [9] and inflammatory infiltrates into tissues [8, 10, 11], improved bone density [12, 13], the acceleration of recovery from tendinopathy [14], sprains [9], and peripheral nerve damage [15, 16], and the reduction of pain [11, 17]. PBM has also been shown to be neuroprotective without adverse effects [18], improve functional outcomes after stroke in rabbits [19] and spinal cord injury in rodents [10, 11], as well as prevent dopaminergic cell loss in Parkinson's disease animal models [20]. Toward the end of 2018, PBM was featured as an innovative option to US Congress for addressing a domestic opioid-use epidemic. Subsequently, the Opioid Crisis Response Act was passed into law which mandates the development and adoption of alternative pain treatments, such as PBM [21, 22]. However, clinical applications and research of PBM requires sound knowledge with simple guidelines about how readily accessible tissues are to specific wavelengths of light.

Many studies have investigated wavelengths near the 670 or 830 nm range, which fall within the 600 to 1100 nm “optical window” for penetrating the skin and underlying tissues [23–25] and which are also centred around the putative range that promotes biological effects [26]. One study that compared a variety of in vivo nervous tissue injuries found 670 nm to be overall more effective compared to 830 nm [27], however, recovery outcomes appear to be highly contingent on the combination of irradiation and injury severity [11, 27]. Furthermore, based on findings in the retina, it has been proposed that irradiation dosage should be adjusted to the severity of injury to achieve beneficial protective effects, but that overdose may be harmful [28]. Thus, while red-light treatment may offer potential benefits for in treating sports-related injuries, such as reducing recovery time [9] and pain [11, 17], to optimise treatment outcomes, it is necessary to determine the penetration parameters for delivery of an appropriate dose, as well as identifying body regions that are accessible to light treatment.

To optimise treatment or conduct research in this field, the delivery parameters of light to targeted tissues must be known, which include the frequency and duration of light exposure, as well as the irradiance of light. However, while all these parameters are easy to control from the light source itself, the quantity of photons that reach the tissue of interest is difficult to establish, and there are currently no clear and simple guidelines that provide information about the penetration of light into different body parts. Once light hits

biological tissues, scattering of photons and absorbance by photoacceptors by different tissue layers attenuate the light. Our main objective here, was to examine the effects of light intensity per unit area (irradiance), skin tone, sex and bone/muscle content on red-light penetration at common sites of sports injuries, and thereby provide a basic guideline for establishing the likelihood of red-light penetration for different body parts. Establishing parameters that ensure red-light is deliverable to a structure of interest is essential to facilitate decisions about red-light treatment paradigms in the clinic, and future research studies. Furthermore, we also aimed to determine whether embalmed cadaveric tissue can serve as an adequate model for predicting penetration in live human tissue, which would be valuable for future studies on regions that are not otherwise accessible in live subjects.

2 | MATERIALS AND METHODS

This study was conducted under the ethics approval of the Human Research Ethics Committee at the Australian National University (protocol no: 2015/288). Nine live human participants were recruited (6 males and 3 females; age = 26.9 ± 2.1 years) and classified into 3 different skin tones (light, medium and dark). Subjects were asked to fill out a questionnaire to determine their Fitzpatrick skin type [29]. Light skin toned participants were all Caucasian (Fitzpatrick skin type scores: 9, 11 and 12); medium skin toned participants were of Iranian, Eastern European and Afghan descent (Fitzpatrick skin type scores: 16, 18 and 23, respectively); dark skin toned participants were of Indian and Sri Lankan descent (Fitzpatrick skin type scores: 25, 30 and 33, respectively). Also included in the study were 17 sites of the limbs, cranium and spinal cord from 7 human cadaver tissues (donation to the Australian National University Medical School Anatomy program; mean terminal age = 81.3 ± 4.3 years). From all subjects, the upper limb sites included the shoulder joint, upper arm, elbow joint, the wrist (distal radioulnar joint), thumb (1st metacarpophalangeal joint) and the middle finger (3rd metacarpophalangeal joint). The lower limb sites included thigh, knee, calf and ankle. At the elbow, knee and ankle, measurements were performed in 2 planes; sagittal (anterior-posterior), and coronal (lateral-medial). In the upper arm and thigh, 2 measurements were performed; one where the light path included the bony structures (humerus, femur), and the other, which was limited to soft tissue irradiation (biceps brachii, hamstring muscles, respectively). Cranium measurements were obtained from human cadavers by placing the light source at the inion or at the bregma and the light probe (described below) was placed on the opposing surface, inside the cranium. Spinal cord measurements were taken by placing the light source on the skin at the junction of the C1 vertebra and the foramen magnum, while the light probe was

placed on the opposing surface of the cervical canal posterior wall.

All measurements were acquired by placing the aperture window (quartz glass, 60 mm diameter) of a custom-built 660 nm LED device directly on the site of interest. The device housed a collimating lens that was placed in front of an array of 50 LEDs (5×10 LEDs; array dimensions, 14×20 mm), which produced a beam that projected 33 degrees (in air) normal to the aperture window. The device produced a spectral peak at 660 nm (Figure 1A) at varying output irradiances ranging from 15 to 500 mW/cm². A light probe tuned to 670 nm (Thorlabs, PM100D) was placed on the opposing side of the respective body part. For cranium and spinal cord measurements, a smaller custom-built 670 nm light probe was used as previously described [11]. Within this light probe, light was detected by a photodiode chip (surface area, 0.62 mm²; maximal response [$>95\%$] to 630–685 nm; ODD-660 W, Opto Diode Corp.) and then amplified (AD8304, Analog Devices). The readings were then calibrated against the Thorlab light probe. There was negligible difference when the tuneable light probe was set at 670 or 660 nm, however, it was necessary to match the readings to the custom light probe which was fixed at 670 nm. Both probes measure the amount of 670 nm light in $\mu\text{W}/\text{cm}^2$. Readings $\geq 0.1 \mu\text{W}/\text{cm}^2$ were considered as

positive for penetration. The thickness of each testing site was measured using digital Vernier callipers. Minimum force was applied to avoid squeezing and multiple measurements were taken to ensure accuracy and consistency. Ambient lights were kept to a minimum and the light probe was positioned so that no positive reading was observed from the ambient light before the LED was switched on. The LED output intensities were increased over the range of 15 to 500 mW/cm² (irradiance: 15, 20, 30, 50, 60, 80, 100, 200, 300, 350, 400, 450, 500; units in mW/cm²) while penetration readings (emitted intensity per unit area) were recorded for each increment.

Statistical analysis was carried out using R (version 3.5.1) [30]. General linear mixed models with Satterthwaite's approximation (LMER; *lmer* function in R) were used for multiple factor analysis accounting for random effects [31], or analysis of variance models (without random factors; AOV; *aov* function in R) where appropriate. Natural log transformations ($\log[x + 1]$) were performed on heteroscedastic data, and all statistical models were validated by inspection of residual plots. Post-hoc comparisons were performed using the *emmeans* function in R with *P*-value adjustments applied using the Tukey method for LMER, or *Tukey HSD* function for AOV. For data that were inappropriate for LMER, a Kruskal-Wallis rank sum test (non-

FIGURE 1 Spectral properties of the red LED light source and site locations. A, Spectral analysis of the LED light source showing the frequency peak at 660 nm. B, Sites of LED light source application (red asterisks) and recording probe (round/oval black circles) are illustrated. Numbers of subjects that demonstrated red light penetration (defined as emitted irradiance measured at the probe $\geq 0.1 \mu\text{W}/\text{cm}^2$) from a 660 nm light source with an irradiance of $\leq 500 \text{ mW}/\text{cm}^2$, and total numbers of subjects tested, are shown for each site and each subject group. For the cranium and spinal cord, readings were measured in cadavers, using a custom-made probe (see Section 2)

parametric test without random factors) was performed, and post-hoc comparisons for non-parametric data were performed using a Wilcoxon rank sum test on raw scores where applicable. *P*-values less than 0.05 were considered statistically significant. All data are expressed as mean $\pm$ SEM unless otherwise stated.

3 | RESULTS

The spectral profile of the LED light source can be seen in Figure 1A. Light penetration readings were acquired from 13 light source irradiances (ranging from 15–500 mW/cm²) in all 9 live subjects, and 2 or 3 of 7 individual cadavers depending on the site. Overall, there were 2395 observations taken for this investigation. From these, 25 individual penetration data points were missing across 5 cadavers: (3 biceps, 2 middle finger, 6 sagittal ankle, 5 coronal elbow, 2 bregma and 4 inion sites), and 3 data points were missing from the thumb site of 3 live subjects.

The proportion of sites that 660 nm successfully penetrated through the entire tissue at ≤ 500 mW/cm² for each body site is summarised in Figure 1B. The sagittal elbow plane was the only site where penetration was possible in some of the live subjects (3/9) and in none of the cadavers (0/3). Complete penetration was observed at irradiances ≤ 500 mW/cm² from the wrist, thumb and middle finger in all live and cadaver subjects, and the cranium in all cadaver subjects. In addition to these structures, the humerus (8/12), biceps (8/12) and coronal (6/11) and sagittal ankle (6/11) were penetrated in at least half of all (live + cadaver) subjects. There was no observed red-light reaching the spinal cord in the cadaver subjects tested (0/2). These observations demonstrate that at irradiances ≤ 500 mW/cm², red-light has the capacity to penetrate the entire thickness of the distal upper limb and cranium in all subjects, and the upper arm and ankle joint in half or more subjects.

3.1 | All hand structures, and most other structures under 50 mm of thickness are readily penetrated by red-light

To observe the effect of tissue thickness and light irradiance on red-light penetration, we plotted penetration against tissue thickness at 100, 300 and 500 mW/cm² for all structures tested in live (Figure 2A) and cadaver (Figure 2B) subjects.

In live subjects, structures with an optical path of less than 50 mm ($n = 27$ individual structures across 9 live subjects) included the wrist, thumb, and middle finger. Increased red-light irradiance significantly elevated penetration of these structures (Figure 2A; $P = 2.0 \times 10^{-14}$, LMER), which were penetrated in 100% of cases by all irradiances ≥ 100 mW/cm². The following models describe the relationship between red-light penetration and tissue thickness for

FIGURE 2 Penetration of red-light in tissues of live and cadaver subjects for 3 source irradiances. A, Penetration from 660 nm light at irradiances of 100, 300 and 500 mW/cm² are plotted against tissue thickness for all structures measured in live subjects ($n = 135$). Statistical comparisons made for tissue thicknesses of < 50 and ≥ 50 mm are indicated for effects of red-light source irradiance. B, Same as shown in A for all cadaver tissues ($n = 42$). Grey dots indicate data from cranium measurements. Statistical tests: LMER (black) and Kruskal-Wallis rank sum test (blue). *** $P < 0.001$, ns $P > 0.41$

the three hand structures in live subjects measuring between 22–50 mm, for three irradiances of red-light input:

$$y = 10^{(6.5 - 0.13x)} - 1 \quad (100 \text{ mW/cm}^2) \quad (1)$$

$$y = 10^{(8.0 - 0.16x)} - 1 \quad (300 \text{ mW/cm}^2) \quad (2)$$

$$y = 10^{(9.3 - 0.17x)} - 1 \quad (500 \text{ mW/cm}^2) \quad (3)$$

where y represents red-light penetration (intensity emitted per unit area at the light probe, μW/cm²) and x represents the tissue thickness (mm) for the range of 22 to 50 mm.

In cadavers, structures with an optical path of less than 50 mm ($n = 23$ individual structures across 7 cadavers) included the cranium, spinal cord, biceps, humerus, hamstring, calf and coronal ankle, in addition to the aforementioned 3 hand structures. Increased red-light irradiance significantly elevated penetration in cadaveric specimens (Figure 2B; $P = 8.3 \times 10^{-4}$, LMER). Of these structures, 65% (at 100 mW/cm²) and 74% (at 300 and 500 mW/cm²) were penetrated by red-light in cadavers, however, like live subjects, all (ie, 100%) of the three hand structures (wrist, thumb and middle finger) were also penetrated in cadavers. While the 3 cranium structures (indicated in Figures 2B and 3) were all penetrated by red-light, the one spinal cord specimen measuring 48 mm was not.

For structures with optical paths between 50 and 95 mm (live subject structures: biceps, humerus, sagittal and coronal elbow, coronal knee, calf, sagittal and coronal ankle; cadaver structures: spinal cord, shoulder, humerus, sagittal and coronal elbow, femur, sagittal and coronal knee, calf, sagittal ankle), a significant increase in red-light penetration was observed in live ($P = 2.4 \times 10^{-4}$, Kruskal-Wallis Rank Sum Test) but not cadaveric structures ($P = 0.41$) as the light-source irradiance was increased (Figure 2A and B, respectively). The proportion of structures from live subjects

FIGURE 3 Minimum irradiance required to penetrate tissues in live and cadaver subjects. The minimum red-light irradiances required for detection through all penetrable structures (as per Figure 1B) in live (colour coded for skin tone) and cadaver (grey) subjects are shown as a function of tissue thickness. Three dark-grey dots identify measurements from the cranium of cadaver tissues. Thick dotted line indicates a piecewise envelope for live subjects; fine dotted line indicates the irradiance (100 mW/cm²) and tissue thickness (50 mm) at the point where the piecewise envelope changes slope. Sample size (*n*) for each skin tone and cadaver subjects are indicated in legend. Statistical comparisons between subjects and skin tones are shown for tissue thicknesses above and below 50 mm (indicated above each bar). ****P* < 0.001, ns *P* > 0.33; LMER

demonstrating complete penetration at 100 mW/cm² was 9% of all structures tested (*n* = 53), 28% at 300 mW/cm², and 43% at the 500 mW/cm². The proportion of cadaver structures demonstrating complete penetration of all structures tested (*n* = 19 individual structures across 6 cadavers) was 11% at 100 mW/cm² and 26% at both 300 and 500 mW/cm² irradiance.

There were an additional 55 structures with thickness between 95 and 150 mm that were tested in the 9 live subjects. These structures included the shoulder, humerus, coronal elbow, hamstring, femur, sagittal and coronal knee, calf, and sagittal ankle. No detectable readings were obtained at 100 and 300 mW/cm² irradiances, however, 5.5% of structures demonstrated red-light penetration at an irradiance of 500 mW/cm². These data indicate: (1) all 3 red-light irradiances reliably penetrate through live tissue structures with thickness of 50 mm or less, (2) increasing the irradiance to 500 mW/cm² increases the penetration, and (3) complete penetration of red-light is negligible in tissues with an optical path greater than 95 mm, even at an irradiance of 500 mW/cm².

3.2 | Sex, but not skin tone affects penetration of 660 nm irradiation in penetrable tissues of live subjects

As melanin is a key photon absorber molecule in the skin [32], our next main objective was to examine the effect of skin tone on red-light penetration. For this, we incorporated 491 observation from live subjects of all tissues and irradiances which resulted in penetration of red-light into a model with fixed (intensity, skin tone, tissue thickness and sex) and

random (tissue site) factors. Both irradiance and sex were the most significant factors (*P* < 2e−16 for each factor, LMER), while skin tone (*P* = 0.064) and tissue thickness (*P* = 0.25) failed to reach significance. We then further restricted our data set to exclude all structures greater than 50 mm, due to the poor light penetration through these tissues (Figure 2A). The identical statistical model, using this restricted data set (364 observations) demonstrated that sex is a highly significant factor (*P* < 2e−16, LMER) for red-light penetration. Under these conditions, tissue thickness was significant (*P* = 0.045, LMER), however, skin tone failed to significantly impact on red-light penetration (*P* = 0.064, LMER). Post-hoc analysis revealed that while light vs. medium skin tones almost achieved a significant difference (*P* = 0.0501, LMER, Tukey post-hoc), there was clearly no significant difference between light vs. dark (*P* = 0.40) and medium vs. dark (*P* = 0.32) skin tones. Further skin tone analysis was performed by substituting the Fitzpatrick score for the skin tone factor in a statistical model. This demonstrated multiple examples where penetration through the darker skin tones was not significantly different to lighter skin tone, for example, Fitzpatrick scores of 11 vs 33 (*P* = 0.36, LMER, Tukey post-hoc), 12 vs 30 (*P* = 0.99), 11 vs 25 (*P* = 0.99) and 16 vs 30 (*P* = 0.90) were not significantly different. Conversely, there were multiple examples where skin tone of similar Fitzpatrick scores was found to be significantly different, for example, 9 vs 11 (*P* = 3.2e−08), 11 vs 12 (*P* < 2e−16) and 30 vs 33 (*P* = 4.8e−15). This indicates that the failure to reach significance mentioned above (*P* = 0.0501) between the light vs. medium skin tones was not due to a type-2 error, but was more likely to result from another factor between these groups.

We then investigated if skin tone could affect light penetration at red-light intensities at the threshold of penetration, that is, at intensities presumably most vulnerable to the effect of skin tone. For all penetrable structures, we plotted the minimum irradiance necessary for red-light to penetrate as a function of tissue thickness for each skin tone (light, *n* = 20; medium, *n* = 14; dark *n* = 16), as well as cadaver tissues (*n* = 22) to enable comparisons of penetration between skin tones as well as between live and cadaver subject (Figure 3). Mixed effects models demonstrated that neither skin tone, (*P* = 0.63, LMER) nor subject (ie, live vs cadaveric tissues) (*P* = 0.39, LMER) had a significant effect on red-light penetration.

3.3 | A red-light irradiance of 100 mW/cm² is sufficient to penetrate tissues of up to 50 mm in thickness

We then applied a piecewise envelope for all tissues that were penetrable at 500 mW/cm² that captures the minimum irradiance required to penetrate tissues at a given thickness. This envelope comprises 2 boundary lines (thick dotted line in Figure 3), one relatively flat and other relatively steep,

that captures all data points from live subjects. The two boundary lines intersect at an irradiance of 100 mW/cm^2 and a tissue thickness of 50 mm ; this point indicates that 100 mW/cm^2 is sufficient to penetrate all tissues (ie, all skin tones and cadaver material) of up to 50 mm in thickness that are penetrable by 500 mW/cm^2 or less, with the exception of one cadaver outlier (a humerus site). At an irradiance of 100 mW/cm^2 or less, and at tissue thicknesses of $\leq 50 \text{ mm}$, there is no significant effect of the different skin tones ($P = 0.77$, LMER) or subjects (ie, live vs cadaver tissues; $P = 0.34$, LMER) on red-light penetration. For the majority of tissues greater than 50 mm of thickness, a disproportionately greater irradiance was required for successful penetration (ie, steep boundary line beyond 50 mm thickness, Figure 3). Skin tone still did not have a statistically significant effect over this tissue thickness range ($P = 0.86$, LMER), however, the live subjects generally had greater penetration than cadavers ($P = 0.044$, LMER), and a sex difference was observed, where males required greater intensities to penetrate tissue structures compared to females ($P = 0.035$, LMER).

3.4 | Effects of skin tone, sex and bone/muscle content on red-light penetration of hand structures

To minimise the effect of tissue thickness when evaluating other factors affecting red-light penetration, we selected the wrist, thumb and middle finger sites for comparisons. We selected these structures because: (1) tissue thickness was not significantly different between live and cadaver subjects (Figure 4A; $P = 0.51$, AOV), live subject of different skin tone ($P = 0.69$) or between sexes ($P = 0.27$); (2) all of these structures from live and cadaver subjects were $\leq 50 \text{ mm}$ and therefore penetrable by red-light; (3) two of these structures are of similar thickness but comprise different bone/muscle content. Light penetration through the wrist, thumb and middle finger at all intensities investigated (452 observations) are shown for each skin tone and cadaver tissues (Figure 4B) and both sexes (Figure 4C). Red-light penetration was not significantly different between live and cadaver subjects ($P = 0.45$, LMER), nor between skin tones in live subjects ($P = 0.91$, LMER), however, penetration was greater in females compared to males ($P = 0.046$, LMER).

We were curious to examine the effect of bone and muscle content on red-light penetration as these 2 tissue types have different optical properties [33]. We first compared the penetration between the thumb and the middle finger, 2 structures that were not significantly different in thickness (Figure 4A; $P = 0.77$, LMER, Tukey post-hoc), but which contain vastly different proportions of bone and muscle content. We found that the middle finger joint, a structure with a greater proportion of bone to muscle compared to the thumb, had significantly more penetration across live and cadaver subjects ($P < 2.2 \times 10^{-16}$; LMER; Figure 4B).

FIGURE 4 Penetration of 660 nm through distal upper limb structures in live and cadaver subjects. Penetration of 660 nm light at varying irradiances was measured through the wrist, thumb and middle finger in live subjects with light, medium and dark skin tones, as well as human cadavers of unknown skin tone (grey). A) Mean $\pm$ SEM and statistical comparisons (ns, $P > 0.05$; *** $P < 0.001$; AOV) of tissue thickness for each hand structure are shown for live subjects of each skin tone and cadaver subjects (sample sizes for each group, n , are indicated). B, All data points for Penetration of 660 nm through the wrist, thumb and middle finger are shown and colour coded according to subject and skin tone (as per figure key). Tissue thickness for each data point is indicated by its diameter (tissue thickness range indicated in figure key). C, The same data points for each hand structure is shown according to sex (colour coded as per the figure key)

3.5 | Effects of skin tone, sex and bone/muscle content on red-light penetration of upper arm structures

We compared penetration of the upper arm through 2 sites; one that passed exclusively through muscle (biceps site) and the other that includes muscle and the long bone (humerus site). Tissue thickness was not different between sexes ($P = 0.30$, LMER). There was no significant difference in

FIGURE 5 Penetration of 660 nm through the upper arm in live and cadaver subjects. Penetration of 660 nm at varying source irradiances was measured through the biceps and humerus in live subjects with light, medium and dark skin tones, as well as human cadavers of unknown skin tone (grey). A) Mean $\pm$ SEM and statistical comparisons (ns $P > 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$; AOV) of tissue thickness for each upper arm structure are shown for live subjects of each skin tone and cadaver subjects (sample sizes for each group, n , are indicated). B, All data points for penetration of 660 nm through the biceps and humerus are shown and colour coded according to subject and skin tone (as per figure key). Tissue thickness for each data point is indicated by its diameter (tissue thickness range indicated in figure key). C, The same data points for each upper arm structure is shown according to sex (colour coded as per the figure key)

tissue thickness among the different skin tone groups ($P = 0.51$, AOV) of the biceps or humerus (Figure 5A) in live subjects, however, the thickness of cadaver specimens was greatly reduced compared to the live tissues for both structures ($P = 1.8e-08$, AOV). Furthermore, the humerus site in live subjects was overall significantly thicker compared to the biceps site ($P = 0.003$, AOV), which was not apparent for the cadaver subjects alone ($P = 0.29$, AOV,

Tukey HSD post-hoc). Red-light penetration was not significantly different between the sexes ($P = 0.12$, LMER), nor between live and cadaver subjects ($P = 0.41$, LMER). However, the humerus site demonstrated greater penetration in live subjects only ($P = 6.94e-04$, LMER) compared to the biceps, despite being significantly thicker.

3.6 | Sex, but not skin tone affects red-light penetration for all structures over 50 mm of thickness in live and cadaveric structures

Finally, as we found consistently strong sex differences for structures < 50 mm (Figure 4C) as well as all penetrable structures in live subjects, but a significant difference between the sexes of the upper arm comparison was not apparent, we examined penetration across all structures of thickness of 50 mm or greater at an irradiance of 500 mW/cm² (126 observations). We found that a significant effect of sex was present across all live and cadaver subjects ($P = 0.0017$, Kruskal-Wallis rank sum test), but this was not due to sex differences in tissue thickness ($P = 0.33$, LMER). The effect was stronger when restricted to the live data set ($P = 2.6e-04$, Kruskal-Wallis rank sum test), and moreover, we confirmed that skin tone also had no effect when using this same analytical approach ($P = 0.16$, Kruskal-Wallis rank sum test), nor was tissue thickness affected by sex ($P = 0.25$, LMER) in the live subjects for tissues > 50 mm.

4 | DISCUSSION

PBM is increasingly becoming popular as an alternative therapy for sports-related injuries [34, 35], and despite recently passed legislation in the United States, which encourages its use as an alternative for pain management [21, 22, 36], there are no standardised treatment parameters at present. Irradiance is a key parameter that influences red-light penetration, and which is affected by numerous factors. Our main aim was to determine clinically relevant parameters for red (660 nm) light irradiation, by improving our understanding of the relationship between light irradiance and tissue penetration under the influence of factors such as tissue thickness, bone/muscle composition, skin tone and sex. Our secondary objective was to compare penetration in live and cadaver tissues so that future studies using cadaver tissues can extrapolate findings to live tissues. The results indicate that in live subjects, irrespective of skin tone, red-light readily penetrates tissues up to 50 mm of thickness at an irradiance of 100 mW/cm² or less. Penetration in live subjects is increased in females compared to males, and in structures with a greater bone/muscle ratio. Cadaver tissues generally appear to require similar levels of light irradiance to that of live tissues to achieve penetration for structures less than 50 mm of thickness, but this could result from the

greater variability of this group. Cadaver structures greater than 50 mm of thickness are slightly less penetrable by red-light compared to live subjects.

Our main finding is that red-light can successfully penetrate up to 50 mm below the skin surface of lean tissues at 100 mW/cm^2 in all live subjects. We also demonstrated that increasing the red-light irradiance beyond 100 mW/cm^2 did not greatly improve penetration, and we therefore propose the use of an irradiance of 100 mW/cm^2 for red-light treatments and future research in tissues of 50 mm thickness or less. There was one cadaver specimen that provided an outlier measure at the humerus site, which had a thickness of 30 mm but required 300 mW/cm^2 before we observed evidence of light penetration. Post-hoc inspection of this specimen's history revealed that it was a cancer patient who was severely emaciated on arrival to the donor program. Furthermore, the cadaver underwent an unusual embalming process which also may have contributed to tissue dehydration resulting in further shrinkage of soft tissues and consequently, an increased tissue density (eg, its biceps measured only 14 mm). It is therefore possible that under different circumstances, the humerus site of this specimen would have measured greater than 50 mm and therefore would have not presented as an outlier.

We provided 3 equations that estimate the penetration of red-light through the 3 penetrable hand structures for 3 source irradiances. These equations demonstrate that: (1) for a given input irradiance, as tissue thickness increases, penetration decays exponentially; (2) the relationship between tissue thickness and penetration differs depending on the source irradiance. These observations can be partly explained by the inverse square law which describes how a point source of energy is decreased (diluted) as a function of distance away from the light source. Also, influencing these observations is the finding that as tissues increase in thickness, penetration is not greatly improved by increasing the irradiance from 100 to 300 mW/cm^2 , thus, the light decay rate differs for each of the input irradiance. We speculate that this latter observation may be influenced by the different relative proportions of constituent tissues for the different structures and the varying thicknesses of each structure across the population.

Much of the PBM literature report dosage in units of J/cm^2 . This unit alone is uninformative for enabling penetration decisions because the Joule is a unit of energy derived from the product of intensity (W) and time (s); the same energy can be delivered by changing either or both of these units (ie, low intensity for a longer period, or a higher intensity for a shorter period). We therefore highly encourage the use of irradiance (ie, mW/cm^2) in addition to the time of exposure when reporting light dosage to include information about penetration.

Our criteria for determining penetration was based on any detectible red-light emitted from the tissue. The

technology, we used enabled light detection through tissues at irradiances as low as $0.1 \text{ } \mu\text{W/cm}^2$. Any observable photons in this range obtained from applying red-light to the opposing tissue surface indicate that most of the light was absorbed, either directly or subsequently following transmission and scattering, by the tissues intervening the optical path. While hard to imagine that red-light would have any biological effect at $0.1 \text{ } \mu\text{W/cm}^2$, it has recently been suggested that neuroprotective effects of PBM could result from circulating protective factors generated from peripheral tissues [37]. We have shown here that 660 nm light with an irradiance of 100 mW/cm^2 generates surplus photons in the order of approximately $50 \text{ } \mu\text{W/cm}^2$ after passing through structures of the hand in live subjects. The remainder (ie, up to 99.95%) of the red-light emitted from the source is therefore potentially absorbed by the intervening tissues. Future studies are yet to confirm whether the therapeutic actions of PBM results from the generation of circulating protective factors [37], or direct exposure at the cellular level, where it has been suggested that the range of 0.5 to 4.5 J/cm^2 of red-light is effective at cellular proliferation and the promotion of wound healing [38, 39]. Therefore, it is difficult to speculate on what therapeutic effects are expected to be delivered by 100 mW/cm^2 in humans without dedicating a study to the specific injury and location. The present study provides valuable starting parameters for embarking on such investigations.

Light irradiance and tissue thickness are 2 important, and perhaps obvious factors governing the level of red-light penetration through human tissues. Increasing the irradiance results in an increase in the delivery of photons to the site, and thereby improves penetration, while tissue layers act as barriers that absorb and scatter the incoming photons and thereby reduce light penetration [32]. Tissue thickness has a nonlinear effect on light penetration because changing this parameter results in the following: (1) a change in the optical path length, and (2) alterations in absorbance and scattering that is dependent on the additional tissue layers and/or layer thicknesses. Tissue thickness readings in the present study could have been influenced by the force applied from the spring-loaded callipers to tissues of different compliances, however, our measuring error is estimated to be within (1-3 mm), and therefore unlikely to greatly affect our overall findings.

We observed greater penetration through the humerus compared to the biceps, and the middle finger compared to the thumb in live subjects. This was despite the humerus was greater in thickness than the biceps, and the middle finger and thumb were of comparable thickness. The main difference in the optical path between the structures compared above is the proportion of bone and muscle content. Compared to bone, muscle has a relatively greater capacity to absorb 660 nm due to the higher content of both deoxygenated and oxygenated haemoglobins [40, 41], myoglobins

[42] and mitochondria [43]. Based on previous reports of the absorbance and scattering coefficients [40, 44], the following order of penetration between 600 and 700 nm (ordered least to most) would be expected for the following tissues: (1) subcutaneous fat, (2) skin, (3) muscle and (4) bone. The absorbance coefficient of bone is 20% to 60% lower than that of muscle, while the scattering coefficient for these tissues are similar [33, 40, 44]. Thus, the additional proportion of bone content at the humerus site and in the middle finger is likely to have improved the level of penetration compared to the biceps and thumb, respectively.

After rigorous experimentation and analysis, we found no effect of skin tone on red-light penetration. Melanin is a key absorbing molecule in the skin and is the primary determinant of skin tone [32], thus, subjects with darker skin tone have more melanin present. Melanin has an absorption spectrum that increases with shorter wavelengths: at 660 nm, melanin absorption is reduced to approximately 55% compared to at 500 nm, and 35% compared to at 400 nm [32, 45]. Compared to skin, the absorption and scattering coefficients of fat are ~50% and 200% greater, respectively, over the 600 to 700 nm range [40]. Therefore, fat content would retard penetration more than skin because the interaction of absorbance and scattering is not linear, and increasing both is likely to synergistically reduce penetration [45, 46]. The melanin-pigmented layer resides in the epidermis measuring within 5 to 100 μm in thickness [47–49]. The absorption coefficients of skin at 660 nm for different Fitzpatrick skin types (II–VI) are between 0.5 and 2 cm^{-1} [47]. This 4-fold difference in absorption between these 2 skin tone extremes therefore represents a negligible difference to penetration retardation for 660 nm compared to a 1 mm layer of subcutaneous fat. Fat content was not measured in our study, however, we recruited lean subjects to minimise any effects of fat. Furthermore, our statistical model incorporated each subject as a random factor which accounts for all unique variations, including the effects of individual fat content. Our findings that 660 nm penetration was largely unaffected by skin tone is therefore consistent with these studies characterising melanin absorption at this wavelength, and suggests that other factors, such as fat content, are more likely to play a significant role.

Red-light penetration was significantly greater in females compared to males overall, and in the hand structures, but not the upper arm. One explanation for the absence of a sex difference in the upper limb is that larger structures, such as the upper arm, have greater levels of fat content compared to leaner structures such as the hand, thereby contributing to greater variability in penetration and resulting in an underpowered data set for the upper arm. As our subjects were all lean, we expect minimal effects of fat on lean structures such as the hand, suggesting that there are sex differences of light penetration unrelated to fat content in those structures. Sex difference in red-light penetration may have resulted from a

variety of factors which were beyond the scope of the present study, such as sex differences in skin [50], muscle [51], bone, hair or other connective tissues and/or vascular supply.

We set out to determine if cadaver tissues can serve as a good model for extrapolating penetration data to live tissues. In structures that were relatively low in muscle content, such as the hand, we found that tissue thickness and light penetration in cadaver structures were comparable to that of live subjects (Figure 4). One possible confounding factor between live and cadaver subjects is the embalming process and medium, both of which could potentially alter the way light is scattered and absorbed within the tissue. As there was no significant difference in thickness or penetration between cadaver and live subjects within the wrist, thumb and middle finger comparisons, we suggest that the embalming process generally had minimal impact on these structures. However, data from the upper arm revealed that body sites with larger proportions of muscle content (ie, the biceps vs humerus sites) resulted in significant reductions in the overall structural thickness of cadavers compared to live subjects (eg, Figure 5A). The reduced muscle bulk is likely to occur from the removal of water content which is expected from the embalming process and/or the refrigeration storage of tissues, however, we must also acknowledge that age- and health-related muscle size and water content differences may exist between the live and cadaver subjects. Reduced tissue thickness would improve penetration due to the reduced optical path length. However, with the exception of reduced water content, the overall matter within tissues of embalmed structures remains similar to live subjects, thus, it is reasonable to expect similar levels of penetration in cadavers when accounting for the reduced optical path length in dehydrated tissues. Another difference between live and cadaver tissues may be the displacement of blood in cadaver tissues which may impact on the penetration. The greater variability of penetration observed in cadaver compared to live structures may therefore have resulted from varying degrees of blood displacement during the embalming process.

The purpose of obtaining measurements in cadavers is to enable light readings that could otherwise not be performed in live subjects. In our study, we quantified the penetration through the cranium and spinal cords of cadavers. Given the minimal muscle content overlaying the cranium, tissue shrinkage is unlikely to have had a major impact on the optical path length, however, this was not the case for the spinal cord measurements where multiple muscle layers were present. Assuming minimal fat content between the skin and the brain, our study demonstrates that 100 mW/cm^2 of red-light is sufficient to at least reach the external layers of the cortex in live human subjects. This is supported by other studies which have shown successful light penetration across the skull and into the parenchyma in live subjects [52] as well as

unfixed cadaver tissues [53]. In live subjects, penetration was shown to be greatly impacted on by scalp-to-cortex distance and any air-filled structures, such as the frontal sinus [52].

How do our findings translate to other wavelengths used for PBM? The heterogeneous constituents of tissues make it difficult to speculate on the penetrability of other wavelengths. For example, as wavelength is increased, the absorption coefficient of melanin decreases, while that of water increases by the same amount [54]. However, the relative proportions of melanin and water are vastly different in human tissues, making it difficult to easily translate the findings from the current study to other wavelengths. Such questions are best answered by dedicated studies of the specific wavelength and tissues in question.

5 | CONCLUSION

In conclusion, our data demonstrate that at an irradiance of 100 mW/cm², 660 nm light can reach a depth of approximately 50 mm below the surface of structures comprising skin, bone, muscle, tendons and ligaments, irrespective of skin tone. While increasing the light irradiance increases penetration, it does not greatly increase the capacity for red-light to penetrate thicker tissues over the irradiances studied. While 50 mm penetration is insufficient to completely penetrate many body parts, it is sufficient to access most of the distal limb structures that are commonly injured by athletes [55], and applying light from multiple sides of the structure would increase photon delivery for larger structures. We provide mathematical models that will provide practitioners and researchers the capacity to estimate red-light penetration for lean structures of interest under 50 mm of thickness. Finally, we show that penetration is mildly better in live subjects compared to cadavers for tissue thicknesses greater than 50 mm, and that penetration parameters for inaccessible regions can be determined using cadaver tissues when accounting for tissue thickness.

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