In vitro tissue culture model validation – the influence of tissue culture components on IPL energy output

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Abstract:

Intense pulsed light (IPL) has been used therapeutically in a number of clinical settings, and has been shown to have a photobiomodulatory effect on connective tissue cells, such as those derived from skin and tendon. *In vitro* cell culture models are essential tools preclinically in investigating such treatment modalities, as they help in optimising parameters for successful treatment. However, as culture system components have been reported to absorb part of the irradiated energy, which in turn has a bearing on the amount of light reaching the cells, it is important to establish specific parameters for the particular *in vitro* model used. This study, therefore, investigates the effect of our tissue culture system components on the IPL energy delivered. Individual wells of multi-well plates were irradiated with IPL at different device settings and under variable culture conditions (e.g., in the absence or presence of cell culture media with or without the pH indicator dye, phenol red), and the energy lost through the culture system determined. Our data demonstrated that the IPL device delivered significantly lower outputs than those published, and energy absorption by the culture equipment would further reduce fluencies delivered to the cell monolayer. Furthermore, energy absorption by media containing phenol red was marginally greater than clear media, and resulted in only a small increase in temperature, which would not be harmful to cells. The use of phenol-red containing media therefore is valid and physiologically relevant when examining light-culture system interactions.

Keywords:

Intense Pulsed Light; **Phototherapy**; **Tissue culture system**; **Light energy absorption**

Blinded manuscript

Introduction

Intense pulsed light (IPL) has been used therapeutically in a number of clinical settings, including the treatment of acne vulgaris, birth marks and scars, as well as for hair and tattoo removal, and in skin rejuvenation and wound healing [1]. It has also been tested for its efficacy in musculoskeletal conditions such as tendinopathy [2,3]. IPL systems deliver polychromatic light ranges between the wavelengths of 530 nm and 1100 nm, which enable the targeting of different chromophores at varying depths in biological tissues [4]. This photobiomodulatory effect is dependent on factors related to light parameters (mainly fluence and wavelength), as well as tissue organisation and composition (such as the absorption spectra of endogenous molecules and their depth in the tissue) [5-10]. However, phototherapy outcomes have lacked consistency, and this has been attributed to the diversity of targets and light parameters [11].

The utilisation of *in vitro* / *ex vivo* tissue culture models are important in investigating light-based therapies, as they provide a better understanding of the molecular mechanisms, which ultimately translates into improved treatment [12-14]. Despite this, limited basic research on the safety and effectiveness of light-culture system interactions has been carried out, and such lack of information can be detrimental to the design of clinical trials [11,15]. In contrast to lasers, which are now widely used in medical applications, IPL is not governed by the standard regulations and specifications that ensure accuracy, safety and efficiency of the device. Furthermore, the importance of checking any optical device for the different parameters it provides has been highlighted, as inconsistencies have been reported between actual measured values and those published by the manufacturer [16]. Therefore, the proper testing of a devise prior to its use in the clinical setting is paramount [17]. As such, the use of optical devices in preclinical research necessitates, as a first step, the validation of their energy output and wavelength spectra; establishing these parameters allows for more accurate interpretation of the results, as well as guiding further refinement to establishing safety and efficacy. Furthermore, in establishing accurate protocols for IPL photobiomodulation in the laboratory setting, it is important to ascertain the absorption and transmittance of light energy by tissue culture equipment and culture media, which have been reported to absorb part of the irradiated IPL energy [14]. In addition, as there is a great variation in the type of equipment and media commercially available, which have a varying effect on the amount of light reaching the cells, it is important to establish specific parameters for the particular *in vitro* model used. For example, it has been demonstrated that in treating cells with an IPL source, energy loss was

lowest in polystyrene well plates and highest in polypropylene tubes [14]. Furthermore, media containing the pH indicator dye, phenol red, produces a specific absorption peak (which is absent from phenol red-free media), and this might have a bearing on the light energy reaching the cultured cells/tissues. Therefore, the main aim of this study was to investigate the effect of our tissue culture system components on delivered energy at varying IPL devise settings.

Materials and Methods

IPL devise energy output

An IPL system (iPulse i300, CyDen Ltd., Wales, UK; features as previously described [18] was used in this study. In order to accurately determine the energy output of the IPL devise in the absence or presence of cell culture equipment, a protocol was developed, based on the methods of Hutchinson et al. 2012 and [2,14]. In summary, a sheet of cardboard was prepared with a circular aperture (2.41 cm^2) cut to match the well bottom diameter of an individual well of a 24-well plate (Corning, UK), and enveloped in foil to act as a light shield; this allowed treatment to be delivered to only one well at time, and to avoid exposure of nearby wells (Figure 1A). The foil-enveloped shield was placed in the centre of the sensor unit stage, and irradiated using the IPL hand-piece held in direct contact (Figure 1B), in order to mimic our IPL cell culture treatment protocols [18]. An energy meter (NOVA II Ophir hand-held optical power and energy meter, Newport Corporation, Wales, UK; accuracy within 3%) was used to record energy output at three distinct fluencies, using the IPL device settings of 10, 15 and 20 J/cm^2 (as stated by the manufacturer) under the following conditions using: (1) the full IPL hand-piece block spot size (i.e. 8.9 cm^2, with no foil shield); and (2) a spot size of 2.41 cm^2, created by the aperture of the foil-enveloped shield. Each measurement was carried out in quintuplicate, and the experiment repeated three times.

Energy loss through the culture system (plastic labware and culture media)

The foil-enveloped shield was placed in the centre of the sensor unit stage and culture wells of 1mL or 1.5mL volume inserted into the aperture (Figure 1B), with or without phenol red-free or phenol-red-containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life Technologies, UK). The culture plate well assembly was then irradiated with the IPL hand-piece using the device settings of 10, 15 and 20 J/cm^2, and the energy output recorded as described above. Each measurement was carried out in quintuplicate, and the experiment repeated three times.

In order to determine the energy loss by the culture system, a regular mathematical formula, based on the Dulong–Petit law for specific heat capacity [19], was used to calculate the change in temperature due to energy absorption by culture plate wells, in the absence or presence of culture media, with or without phenol red. Calculations were based on the following assumptions: (a) all energy lost by the culture system was converted to heat, thus producing a measurable temperature change; (b) the specific heat capacity for DMEM media with or without phenol red was similar to that of blood and water, respectively; and (c) as the IPL pulse duration is relatively short (60 ms), there was negligible heat loss during the pulse.

Culture media absorption spectra

A spectrophotometer plate reader (POLARstar Omega; BMG Labtech, UK) was used to measure the absorption spectra of culture media samples (DMEM with or without phenol red), along with phosphate buffered saline (PBS) and distilled water, as controls. Absorption spectra in the wavelength range of 220 nm-850 nm were recorded, with 2 nm resolution [14]. Importantly, a portion of this wavelength range (i.e. 600 – 900nm) lies within the IPL spectrum (530 – 1100 nm). Aliquots of test media, PBS or distilled water (300µl) were pipetted in triplicate into Greiner F-bottom 96-well plates (Sigma-Aldrich, UK) and the optical density absorbance readings recorded. All experiments were repeated three times.

In order to calculate the energy absorption attributable to phenol red within the culture media, its absorption spectrum was extrapolated against IPL emission spectrum data (wavelength vs. optical density; supplied by Dr Mike Kiernan, CyDen Ltd., Wales). Briefly, using the IPL spectrum data, the optical density data for every IPL wavelength unit was converted into a percentage of the total area under the IPL curve, using mathematical formulas. These data were then used to calculate equivalent energy percentages using the total IPL energy delivered (i.e., 17.58, 26.14 and 38.44 J were delivered when the IPL devise was programmed at 10, 15 and 20 J/cm^2, respectively). Similarly, each wavelength unit of the phenol red spectrum was converted into a percentage of the total area under the optical density curve (**Error! Reference source not found.**). Using these datasets, the energy absorbed due to the presence of phenol red was subsequently extrapolated.

Statistical analysis

Data were analysed using Microsoft Excel (2010), IBM SPSS 20 statistics and GraphPad Prism 5 software, and presented as graphs or tables using means plus or minus the standard error of

the mean. Parametric data were tested with one-way ANOVA (analysis of variance) to compare means, whilst a Kruskal-Wallis H-test was used as a non-parametric test. Post-hoc tests were used for multiple comparisons in case of significance based on the homogeneity of the variances. Significance was determined at a p-value less than 0.05.

Results

IPL devise output fluence

Analyses revealed that the IPL handpiece output fluence measurements (spot size 8.9 cm^2) were 6.12, 10.20 and 14.16 J/cm^2 at the IPL device settings of 10, 15 and 20 J/cm^2, respectively (Table 1). This represents an average of a 33% deviation from the manufacturer's published IPL output settings, with the greatest discrepancy occurring at the lowest IPL setting (with an IPL output of 61% of the device setting).

In addition, data revealed that the IPL output measurements, using a spot size of 2.41cm^2 (created by the aperture in the foil-enveloped shield), were 7.29, 10.85 and 15.95 J/cm^2 at the IPL settings of 10, 15 and 20 J/cm^2, respectively; this represents an average of 75% of the devise settings (Table 1). Furthermore, by inserting a plate well into the aperture, fluencies were further decreased by an average of 10.5%. In both cases (i.e., with or without a plate well bottom), the greatest parity between published IPL settings and those measured occurred at the highest IPL setting.

Energy loss by the culture system

Analyses revealed that in comparing energy loss across the culture system (Figure 2A), empty wells alone accounted for a high proportion of the total (approximately 50–75%). Furthermore, in culture wells containing media with or without phenol red, there was a significant increase in energy loss versus empty wells alone, when comparing equivalent volumes and delivered energies ($p < 0.05$, Mann–Whitney U test). Similarly, higher well volumes (i.e., 1.5 versus 1 mL) were associated with a significant increase in energy loss overall, whilst the delivery of higher energy levels (26.14 and 38.44 J) resulted in a significantly reduced loss (approximately 5-10%) compared with the lowest IPL energy ($p < 0.05$, Mann–Whitney U test).

Interestingly, in comparing energy loss values for wells filled with clear or phenol red-containing DMEM, data revealed that phenol red is responsible for an additional energy loss ranging from 5.43 to 7.61% (Figure 2B). Moreover, the energy loss due to phenol red in a 1mL

volume remained relatively constant when comparing the various energies delivered, and was not statistically significant ($p > 0.05$, Mann–Whitney U test). In contrast, delivering the highest energy level (38.44 J) to a 1.5 mL volume resulted in a significantly increased loss compared with delivery of 17.58 or 26.14 J ($p < 0.05$, Mann–Whitney U test).

The temperature change through the culture system when irradiated with IPL energy was also evaluated (Figure 2C). These data reveal similar trends to those examining energy loss within the system (Figures 2A & 2B); for example, in comparing values for empty versus DMEM-filled wells, unfilled wells are responsible for a high proportion (approximately 60%) of the temperature change. In addition, phenol red-containing media was associated with a significantly increased temperature change versus clear media when comparing equivalent volumes and delivered energies (Figure 2C), whilst there was no significant change in temperature when comparing 1mL or 1.5 mL volumes of phenol red DMEM ($p > 0.05$, Mann–Whitney U test). Analyses also revealed that the delivery of higher energies were associated with a significantly increased temperature change, whereas higher well volumes (1 mL versus 1.5 mL) were associated with a significantly reduced temperatures change when comparing equivalent wells ($p < 0.05$, Mann–Whitney U test).

Culture media absorption

Analyses revealed that media samples containing phenol red produced a distinct absorption spectrum spanning 350 nm-602 nm, with an absorption peak at approximately 560 nm, and OD value of 0.53 OD units (Figure 3A). In contrast, the OD values for phenol red-free DMEM, distilled water and PBS were generally very low over the 350 to 850 nm range. The effect of DMEM with or without phenol red on the transmittance spectrum following irradiation with IPL at a fluence of 15.9 J/cm^2 was next evaluated (Figure 3B). Phenol red-containing media manifest a low level of transmittance (i.e. increased absorption) over the 530-600nm range, which equated to an approximate 11% energy loss with delivery of 38.5 J.

Discussion

An important step in developing *in vitro* cell culture models to investigate the effects of light therapy is validation of the optical devise itself within the system. In doing so, our results showed that during irradiation of the plate wells, the IPL device delivered an average of 75% of the published outputs across the three devise settings (i.e., when the IPL device was programmed at 10, 15 and 20 J/cm^2, the fluencies recorded were 7.29, 10.85 and 15.95 J/cm^2

respectively (Table 1). A further decrease (by an average of 10%) occurred due to the presence of the plate well bottom, implying that the actual fluencies delivered to the cell monolayer would be approximately 65% of the recorded output. Previous studies have also demonstrated that measurements of energy output were less than those published by manufacturer [16]. In addition, our data showed an increase in the energy recorded with decreased IPL handpiece spot size, indicating that IPL output is more focussed at the centre of the handpiece and / or more energy is scattered at the periphery.

Another important aspect of the application of IPL in *in vitro* studies is to consider the effect of energy loss through the culture system, as this will influence the effectiveness of the treatment itself. Firstly, as part of the emitted IPL wavelength spectrum is absorbed by the culture equipment, it will be unavailable to the cells or tissue explants; and secondly, as IPL energy passes through the system, a proportion of it will be converted into heat, potentially raising the temperature of the culture media, and affecting the viability of the cells or explants. Indeed, our experiments showed that the equipment alone (i.e., culture plate wells) were responsible for more than 50% of the energy lost through the system, with the addition of culture media (with or without phenol red) further increasing energy loss (Figure 2A). This can be explained by IPL light-culture system interactions which include refraction of light as it passes through air, plastic and media with their differing refractive indices, as well as absorption, reflection and scattering effects, as light interacts with the culture components [20]. Furthermore, our data demonstrate that higher culture plate well volumes (i.e., 1.5 versus 1 mL) are likely to increase light interactions within the system (e.g., reflection, absorption, scattering and refraction), resulting in an increased energy loss overall.

Importantly, phenol red-containing media further increased energy loss by between 5.4% - 7.6% versus clear media (Figure 2B); this can be attributed predominantly to energy absorption by phenol red itself, which manifests a major absorption peak at 560 nm, together with a corresponding drop in transmittance within this wavelength range when irradiated with IPL (Figures 3A & 3B). In contrast, the absorption spectrum for clear DMEM was very low (with corresponding increased levels of transmittance compared with phenol red-containing DMEM), suggesting that energy loss through this media is mainly due to scattering, reflection and refraction (and to a much lesser extent absorption). Our data are consistent with published absorption spectra for culture media with and without phenol red [14].

As the IPL light passes through the culture system, part of its wavelength will be absorbed and converted into heat. In order to investigate this further, we examined the temperature change that occurred within the system with IPL irradiation (Figure 2C). Our data demonstrated that the maximum calculated temperature change was 2.5^{o}C (assuming that all energy lost is converted into heat), which was associated with phenol red-containing media at the highest fluence delivered. Therefore, based on the knowledge that human / mammalian cells and explants are routinely cultured at 37^{o}C for optimal growth [21] , then the maximum temperature that the culture media could reach would be 39.5^{o}C, which has been proven to be unharmful to cells. For example, it has been reported that fibroblast cells can survive at 40^{o}C for more than 25 passages [22]. Furthermore, tendon fibroblasts have been shown to survive high temperatures (up to 45^{o}C) for durations of up to 1 hour, with minimal reduction in viability; this is consistent with the physiological situation, where tendon cells withstand prolonged periods of exercise during which tendon temperature is increased [23]. Our studies have also shown that tendon cell viability increased in the presence of phenol red when treated at the highest IPL energy [18], which may be attributed to a photobiostimulatory effect through thermal reaction [9,10]. Indeed, using our in vitro model, we demonstrated that IPL was not detrimental to tenocyte morphology, migration or viability under any of the conditions tested [18].

Taken together, these data validate the use of phenol red-containing media in tissue culture systems examining the effects of IPL as: firstly, the temperature of the media remains lower than the hyperthermia threshold (42-45^{o}C) that leads to cell death [24,25]; secondly, the absorption spectrum of phenol red (which peaks at 560 nm) constitutes a minimal loss of the effective IPL wavelength spectrum, which has optical window of 600 – 900 nm [26,27,24,10,28]; and thirdly, the presence of phenol red mimics normal physiological conditions, with a similar absorption spectrum to that of blood [29,30].

Conclusion

This study highlights the importance of testing an optical device within a cell culture model system, as outputs may differ to those published by the manufacturer. Furthermore, the use of phenol-red containing media is valid and physiologically relevant when examining light-culture system interactions.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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Hashemite University of Jordan is the sponsorship for the corresponding author during his PhD degree and this study was part of his PhD thesis.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

No informed consent is needed (in vitro study)

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Table 1: Measurement of IPL devise output fluencies, using a spot size of 8.9 or 2.41 cm^2 at different device settings, with or without well a plate bottom; SEM: standard error of the mean.

IPL devise fluence setting (J/cm^2)	Spot size – 8.9 cm^2		Spot size = 2.41 cm^2			
			No plate well bottom		With plate well bottom	
	IPL output (J/cm^2) ± SEM	IPL output (%)	IPL output (J/cm^2) ± SEM	IPL output (%)	IPL output (J/cm^2) ± SEM	IPL output (%)
10	6.12 ± 0.13	61.17	7.29 ± 0.10	72.94	6.11 ± 0.13	61.09
15	10.23 ± 0.20	68.21	10.85 ± 0.06	72.30	9.41 ± 0.10	62.72
20	14.16 ± 0.16	70.80	15.95 ± 0.08	79.74	13.92 ± 0.13	69.61

Figure legends

Figure 1A: Energy meter showing hand display unit (left) and sensor unit (right) with 1 mL plate well assembled in the foil-enveloped shield (yellow arrow). 1B: Diagram showing energy absorption testing process; arrows represent the IPL pulse direction and a plate well (red) assembled in the foil-enveloped shield (grey)

Figure 2: Loss of IPL energy within culture plate wells (1 or 1.5 mL), (A) in the absence or presence of culture media, with or without phenol red at different delivered energies; (B) due to the presence of phenol red, relative to total energy delivered; and (C) presented as maximum temperature change (equivalent to energy lost by the culture system) within culture plate wells

Figure 3: Absorption and transmittance spectra of DMEM with and without phenol red, (A) in comparison with PBS and distilled water (absorbance); and (B) when irradiated with IPL at a fluence of 15.9 J/cm^2.

Figures

Figure 1

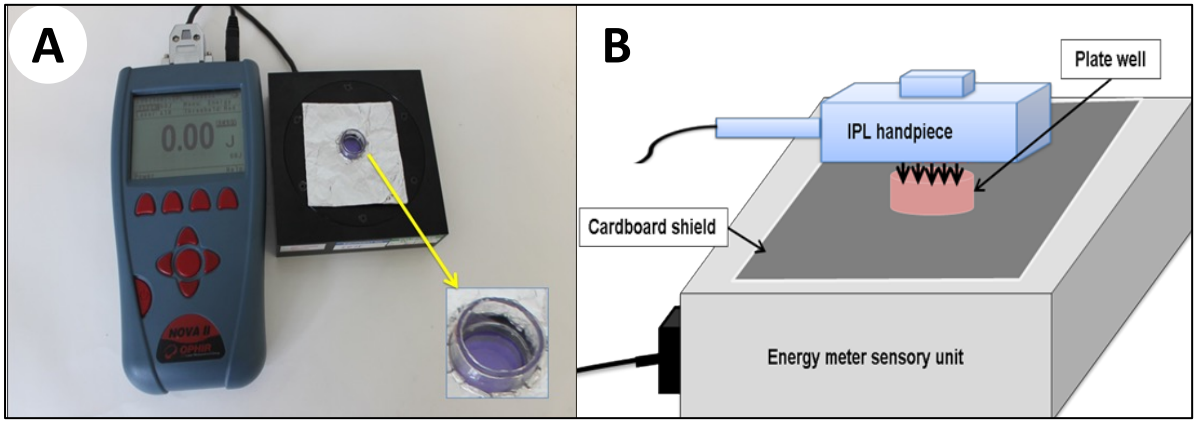


Figure 2A

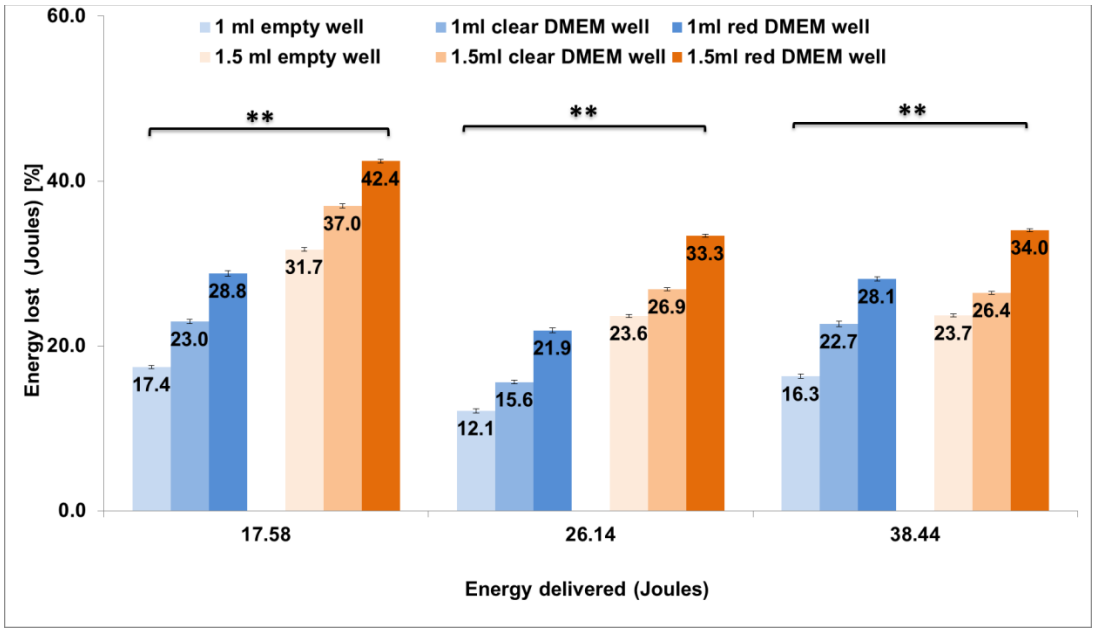


Figure 2B

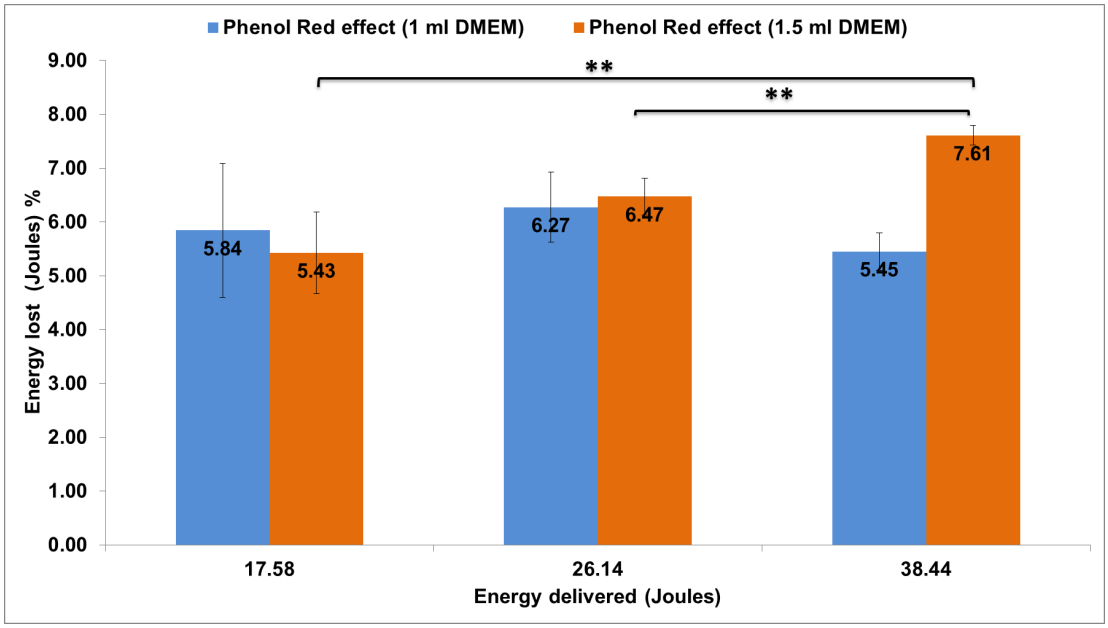


Figure 2C

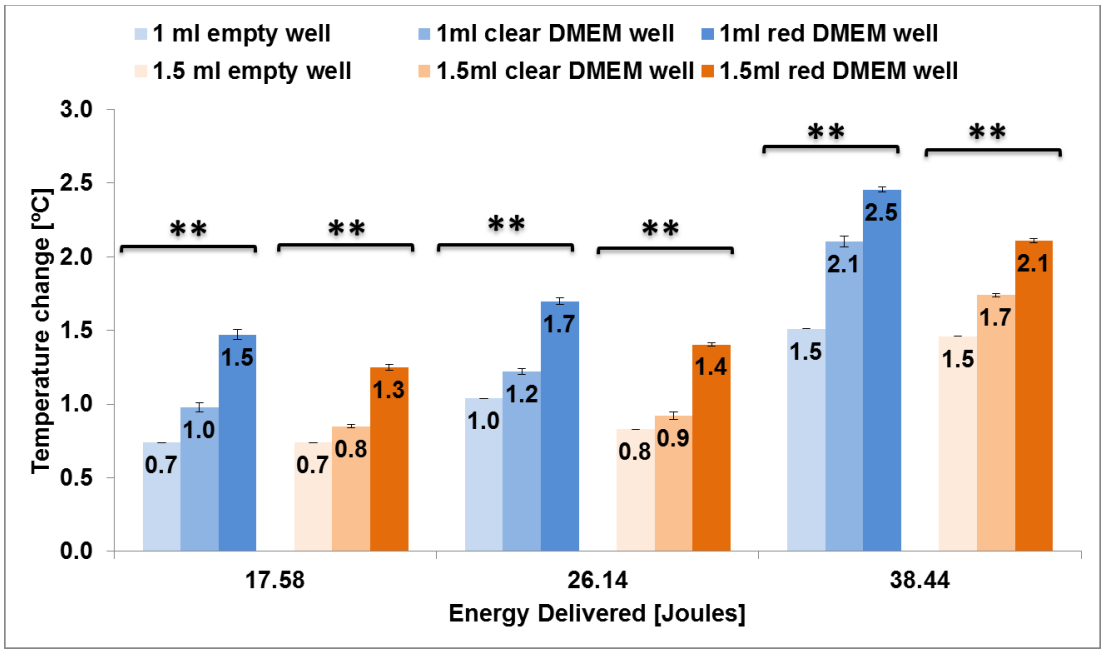


Figure 3A

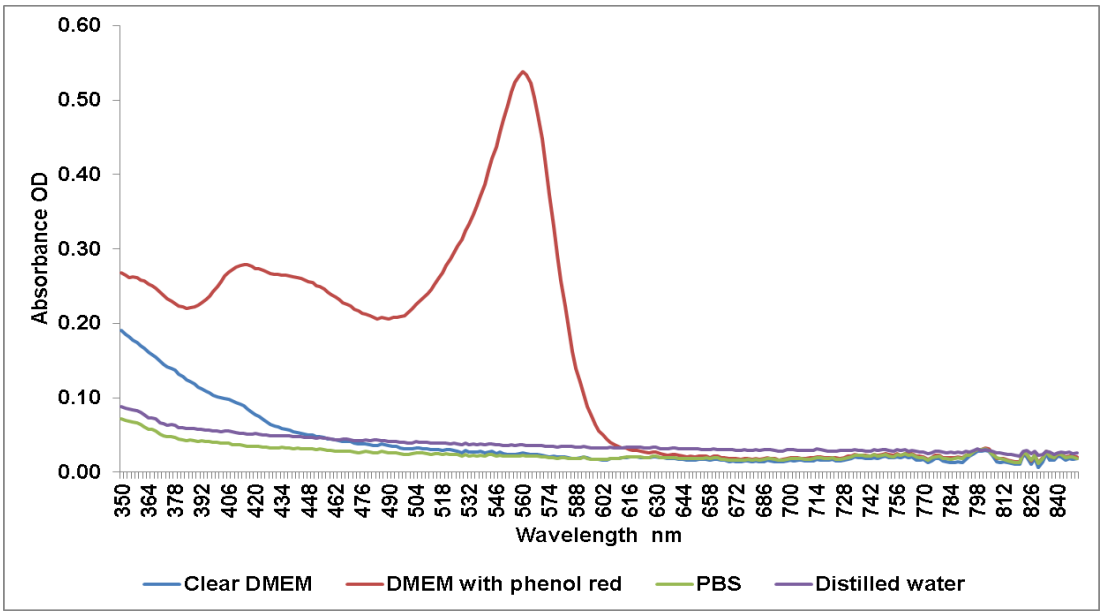
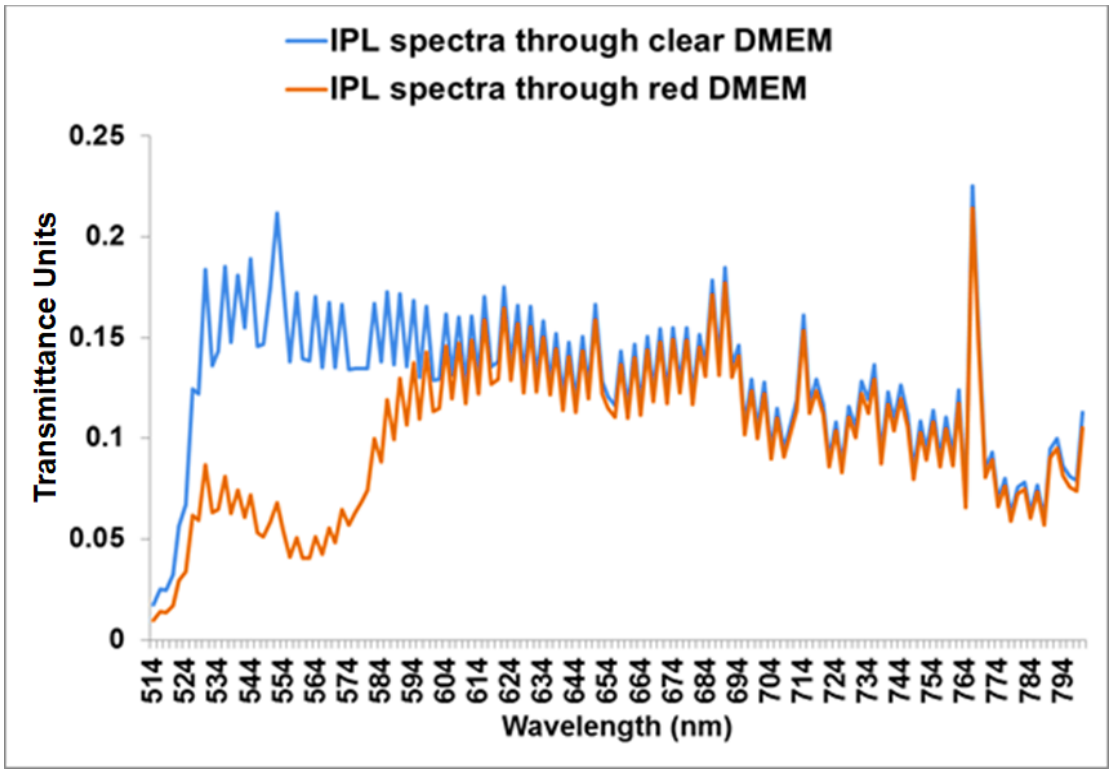


Figure 3B


IPL spectra through clear DMEM
IPL spectra through red DMEM
Transmittance Units
0.25
0.2
0.15
0.1
0.05
0
514
524
534
544
554
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574
584
594
604
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Wavelength (nm)