To: Dr Pieter Neethling
SAIP Sub-editor: Photonics.

From: Prof N. Houreld
Date: 14/11/2022
Title: Photobiomodulation at 830 nm modulates proliferation and migration of wounded fibroblast cells
Ref: ID 096

Corrections made to manuscript ID 096

Dear Dr Pieter Neethling

We appreciate your decision to consider our paper for the SAIP conference proceedings (ID 096). We welcome the opportunity to reply to reviewer's comments. Changes in the manuscript has been written in red.

REVIEWER #1:

1. Please supply references for statements such as "The prevalence of chronic wounds are rising…"
   Author Response: References have been added.

2. Methodology: You state that samples were analysed three times-did you analyze the same samples three times or did you analyse three different experiments? Please state the n-values and number of technical repeats per n-value. Please also state the passage of cells that were used for these experiments. For all your results, you state n=3. Are this three technical repeats? Please state how many images were used to calculate the distances for the migration assay?
   Author Response: We analysed 3 different experiments for each model. This has been clarified in the proceeding pg 3 to "Experiments were performed three times (n=3),…"
   The passage of the cells has been added to pg 2 "… between passages 8 and 11 were utilized…".
   Three images from 3 different experiments were used to calculate the average distance for the migration assay. This has been included in the proceeding pg 2 "The distance between the wound margins was measured from three experimental repeats and used…"

3. Conclusion: Please provide some explanation or suggestion for why irradiation will significantly decrease cell proliferation in NW cells at 24 hours, but increase it at 48 hours?
   Author Response: This may show that at 24 h after irradiation PBM had no effect on the proliferation of normal cells and may suggest that extended incubation post-irradiation is necessary to reap the full effects of PBM on cell proliferation. This has been included to the conclusion (pg 5)

"Our results demonstrate that at 24 h after irradiation PBM had no effect on the proliferation of normal cells and may suggest that extended incubation post-irradiation is necessary to reap the full effects of PBM."

4. You seed and grow your cells to absolute confluency. Might this impact your results? Please state why you use this seeding concentration.

   Author Response: The seeding density for experiments has been previously optimized and used in numerous studies on WS1 cells. Cells are seeded to confluency to enable a central scratch in a confluent monolayer to be performed. A higher seeding density for flow cytometry is used (10^6) as per manufacturer protocol. Articles have been published from the centre using the same seeding density for the same assay.

REVIEWER #2:

1. The authors state the following with regards to the light source "This therapeutic technique uses light with distinctive properties of coherence (light waves in temporal and spatial phases), collimation (rays are parallel and travel in a single direction without divergence), and monochromaticity (single wavelength radiation) [4]" This statement is in direct contrast to the following, taken from the conclusion of reference [4]. "The current total evidence appears to support the idea that photobiomodulation is not dependent on lasers or coherence, but quasimonochromatic LED devices and even broad-wavelength light sources such as water- filtered infrared-A can also yield physiological effects. The comparisons between lasers and LEDs lend support to this idea" [4] Heiskanen V and Hamblin M R 2018 Photochem. Photobiol. Sci. 17 1003-17

   This statement therefore requires amendment or removal as from literature that is cited it would seem that LED's would work as well as lasers.

   Author Response: The statement in the introduction (pg 2) has been changed to "This therapeutic technique is a non-invasive, non-thermal therapy that involves the application of low-powered light sources such as lasers, light-emitting diodes (LEDs), and broadband light using appropriate filters, in the visible light (400- 800 nm) and infrared (760- 1400 nm) spectrum to promote tissue regeneration [5,6]."

2. Table 1: Check these parameters again. 114 mW on a spot size of 9.1 cm^2 gives an intensity of 12.53 mW/cm^2. An irradiation time of 364 s would yield a fluence of 4.56 J/cm^2 and not 5 J/cm^2. 4.56 J/cm^2 over 9.1 cm^2 yields a delivered energy of 41.5 J so this seems correct. The correct value should be changed in the text throughout.

   Author Response: The correct values have been added.

3. Claims are made that after 24 h there is a significant increase in the cell migration of the irradiated cells compared to the control. More information on the statistics is required in order to support this claim. Mention is made of a (n=3). Were these 3 different samples or 3 measurements o the same

sample? Looking at the scratches on the micrographs after 24 and 48 hours, the scratches definitely don't have sharp edges anymore which will introduce considerable error in the measurement of the width of the scratch. How was this accounted for in the analysis? At how many positions were the width measured and how were these positions determined? How representative are these micrographs across the complete sample? All these questions need addressing in order to be able to make the claim that the measured change in rate is significant.

Author Response: The distance between the wound margins was measured from three experimental repeats (n=3) i.e. on three different samples. This has been clarified on pg 2. Cells on the edge of the wound margins migrate towards the central scratch to close the newly created gap. Cells will form multi-polar projections until cell-cell contact is established. This is very characteristic of fibroblasts, and smooth edges will not be obtained while cells move towards each other to close the gap. To some degree, the central scratch assay mimics *in vivo* cell migration (Liang *et al.,* 2007). The same position on each plate/model was measured over the 48 h period. The position on the plates were marked and the position on the XY axis on the microscope noted. This has been included in the proceeding.

The central scratch assay is a widely used accepted model for measuring cell migration and an accepted model to study wound healing *in vitro*. This model serves as a valuable tool for understanding the effect of the experimental assays for promoting wound healing. It is cost effective and involves a simple experimental design thereby making it an invaluable method for understanding cell migration favoring the quantification of cell migration under controlled experimental conditions.

Liang CC, Park A, Guan JL. *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. *Nat Protoc*. 2007;**2**:329–333. doi:10.1038/nprot.2007.30.

Martinotti S, Ranzato E. Scratch wound healing assay. Epidermal Cells. 2019;2:225–229.36.

Walter MNM, Wright KT, Fuller HR, MacNeil S, Johnson WEB. Mesenchymal stem cell-conditioned medium accelerates skin wound healing: an in vitro study of fibroblast and keratinocyte scratch assays. Exp Cell Res. 2010;316:1271–1281. doi:10.1016/j.yexcr.2010.02.026

4. Scale bars on micrographs are not legible. I realize it is indicated in the label but tit can also be increased on the images themselves.

   Author Response: Scale bars have been increased.

Kind Regards,

Prof N. Houreld

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