UNIVERSITY OF JOHANNESBURG

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

From: Dr Sandy W. Jere
Date: 22-11-2022
Title: Photobiomodulation at 660 nm reduces stress-induced apoptosis in diabetic wounded fibroblast cells in vitro
Ref: ID 044

Corrections made to manuscript ID 044

Dear Dr Pieter Neethling

We appreciate your quick decision regarding our paper for the SAIP conference proceedings (ID 044). We, however, still think our study is fit to be considered for publication. Most of the reviewer comments are based on the model used and the efficacy and validation thereof. This 'diabetic' model has been used for over 12 years in the research centre and previous published works have shown that differences between hyperglycaemic and normoglycemic models at the concentration of glucose used is effective. The model used in the proceeding was referenced. Also, the reviewers misinterpreted the methodology, which stated that cells were grown in an additional 17 mM glucose, thus the total glucose concentration was not 17 mM, but in fact 22.6 mM. Also, PBM treated diabetic cells were compared to unirradiated diabetic control cells, clearly showing that PBM reduced apoptosis. Thus to say it is difficult to evaluate if apoptosis was decreased with PBM is unfounded.

We have addressed and replied to all the reviewer's comments, and changes in the manuscript has been written in red. Please kindly reconsider our paper for publication.

REVIEWER #1:

1. The study investigates whether photobiomodulation reduces cell death in fibroblasts cells treated with a higher glucose concentration. The language and grammar use overall is good and the manuscript is well-structured. However, the model lacks validation and the methodology is unclear. The study propose that oxidative stress, due to hyperglycemia, is responsible for diabetic wound cell death through apoptosis. The model use 17 mM glucose, although the published and physiologically appropriate concentration is 33 mM. The study did not evaluate oxidative stress, nor included untreated cells as a negative control, and it is therefore difficult to evaluate whether apoptosis was decreased with PBM or not.

Author Response: These cell models and the D-glucose concentrations used in this study have been tested and used for more than 12 years at Laser Research Center (LRC). The center has published studies that have shown clear differences between cells grown under hyperglycemic conditions (diabetic) and cells grown under normal conditions (normal), and this has been referenced. A hyperglycemic condition, 'Diabetic', was created by continuously growing cells in complete media (with a basal glucose concentration of 5.6 mM) with an additional 17 mM glucose, and a total D-glucose concentration of 22.6 mM. Many other studies from other research groups have been published using such in vitro models with concentrations of glucose in similar ranges i.e. Vinck et al 2005, and Iannello et al 2005. The total glucose concentration of 22.6 mM has been added to the proceeding for further clarity, but should also be read in conjunction with the cited references.

A study by Rajendran et al 2021, evaluated oxidative stress in cells grown at the same glucose concentration that this study used (total D-glucose concentration of 22.6 mM). They noted that PBM progressed diabetic wound healing by attenuating oxidative stress and inhibition of the FOXO1 signalling pathway. The present study aimed at evaluating if photobiomodulation at 660 nm reduces apoptosis (that come about due to oxidative stress in hyperglycaemic environment) in diabetic wounded fibroblast cells in vitro. Due to the limited space and page limits, not all methods could be explicitly added, analysed and explained. In this regard, most details have been referenced.

2. Abstract: Apoptosis results in cell death and can therefore not sustain development nor homeostasis. Please correct this sentence.

Author Response: The sentence "Oxidative stress provokes apoptosis, a programmed cell death that typically sustains the developmental mechanism for normal body homeostasis" was meant to mean that in normal circumstances apoptosis sustains the developmental mechanism for normal body homeostasis. To make it clearer to the reader, a comma has been added to the sentence, and now reads as "Oxidative stress provokes apoptosis, a programmed cell death, that typically sustains the developmental mechanism for normal body homeostasis". Apoptosis to some degree is an important requirement in wound healing processes, it is essential in eradicating unwanted cells and advances the development of granulation tissue, and thus is important in normal body functioning.

3. Introduction: Oxidative stress occurs due to unregulated ROS production, not anti-oxidant production. DM is not caused by oxidative stress. It is a metabolic disease that develops due to both environmental and genetic factors. Although oxidative stress can result in altered immune responses or DNA damage that can lead to cancer, oxidative stress alone does not cause the disease listed here. Please write an abbreviation out the first time it is used, i.e PBM in the title.

Author Response: The sentence has been misinterpreted "Oxidative stress comes about due to disproportional production of ROS and antioxidants" As the reviewer indicates, oxidative stress does not come from antioxidant production, but as indicated in the sentence it comes about due to the

disproportional production of ROS and antioxidants, meaning there is increased ROS and decreased antioxidant production.

The authors agree, oxidative stress does not cause diabetes, and what was meant here was that increased oxidative stress leads to the development of diabetic complications, which is indicated further down in the text. The sentence has been corrected in the text and DM has been removed. The sentence now reads as "…over time, oxidative stress can contribute to diseases including atherosclerosis (hardening of the blood vessels)…". The sentence that read as "..evidence shows that oxidative stress plays a major role in the pathogenesis of DM and its devastating complications." has also been changed and now reads as "..evidence shows that oxidative stress plays a major role in the development of DM complications [5]."

4. P2, 2nd paragraph: please provide reference for statement that hyperglycemia diminish insulin secretion and action.

Author Response: Reference has been added.

5. Please provide ref for next sentence re decreased anti-oxidant activity and DM-wound healing.

Author Response: Reference has been included.

6. Please provide ref for "In progression of wound healing…"

Author Response: Reference has been added.

7. Please remove 'involuntary'. Apoptosis is often described as cellular suicide and is the result of a highly coordinated cellular response. Except for one commentary (Padock, 1999, PNAS) apoptosis is not regarded as involuntary.

Author Response: The word 'involuntary' has been replaced by 'programmed'.

8. Please provide a ref for: PBM is a process that is initiated…. Please write out abbreviations the first time it is used in text and it is good practice not to start a sentence with an ebbreviation.

Author Response: PBM has been written out in full in the abstract. Due to the limited space and page limit (6 pages) abbreviations have been used consistently throughout whether at the beginning or middle of a sentence. Reference as suggested has been added.

9. Please provide a ref for: The mitochondrial cytochrome c oxidase absorbs… Please state at what wavelength Cyt C absorbs light and if this corresponds with the 660 nm used in this study.

Author Response: PBM has been shown to be absorbed by and effect COX at red and NIR wavelengths. D. Pastore, M. Greco, S. Passarella (2000) Specific helium-neon laser sensitivity of the purified cytochrome c oxidase, International Journal of Radiation Biology, 76:6, 863-870, DOI: 10.1080/09553000050029020, showed that the purified enzyme, cytochrome c oxidase was activated in vitro by red laser (633 nm). It is a well-known theory, and shown in numerous studies that red and near-

infrared light is absorbed and affects cytochrome c oxidase. The wavelength of light used in this study, 660 nm, is visible red light, and a previous study has shown that 660 nm affects cytochrome c oxidase activity leading to increased mitochondrial activity and ATP synthesis (*Houreld, N.N., Masha, R.T. and Abrahamse, H. (2012), Low-intensity laser irradiation at 660 nm stimulates cytochrome c oxidase in stressed fibroblast cells. Lasers Surg. Med., 44: 429-434. https://doi.org/10.1002/lsm.22027*). Other papers have also indicated that red and NIR light affects cytochrome C oxidase (*Hamblin, Michael R. "Mechanisms and Mitochondrial Redox Signaling in Photobiomodulation." Photochemistry and photobiology vol. 94,2 (2018): 199-212. doi:10.1111/php.12864*; *Passarella, Salvatore, and Tiina Karu. "Absorption of monochromatic and narrow band radiation in the visible and near IR by both mitochondrial and non-mitochondrial photoacceptors results in photobiomodulation." Journal of photochemistry and photobiology. B, Biology vol. 140 (2014): 344-58. doi:10.1016/j.jphotobiol.2014.07.021*) In this regard, the sentence 'The mitochondrial cytochrome c oxidase absorbs…' has been changed and reads 'The clarification of the mechanism of PBM remains elusive. However, it is suggested that its effect is influenced by the fluence, power, wavelength and period of application. A range of photoreceptor proteins in the mitochondria and other cell partitions including cytochrome c oxidase absorb the light energy and convert it into photochemical energy resulting in increased adenosine triphosphate (ATP) production, protein synthesis and cell proliferation' Reference has been added [13].

10. This work was published previously in 2012-please state what the novel aspect is that is being investigated in this study.

Author Response: No paper published on apoptosis and cell survival in 2012 in the same cells, models and wavelength could be found. A paper on collagen production and proliferation, and effect on cytochrome c oxidase was published, but not apoptosis. In 2013 Houreld and Abrahamse published a proceeding, but caspase activity was not done at a wavelength of 660 nm, but rather at 636 nm. The study by Rajendran et al, 2021, assessed the attenuatiation of oxidative stress by red and near infrared PBM on diabetic wound healing through the inhibition of the FOXO1 signaling pathway. The present study aimed at evaluating if PBM at 660 nm can reduce apoptosis in diabetic wounded fibroblast cells in vitro.

11. Methodology: Please state the standard conditions in which these cells are cultured as well as the glucose concentration of the standard media that is used. Please provide an alternative reference for the hyperglycemic conditions. The standard, published, physiologically translatable hyperglycemic cell culture concentration is 33 mM glucose. Please state how hyperglycemia was validated in your model? I e did you look at insulin resistance, markers of glucotoxicity, ROS?

Author Response: These cell models and the D-glucose concentrations used in this study have been tested and used for more than 12 years at Laser Research Center (LRC). The center has published studies that have shown clear differences between cells grown under hyperglycemic conditions (diabetic) and cells grown under normal conditions (normal), and this has been referenced. A hyperglycemic condition, 'Diabetic', was created by continuously growing cells in complete media (with a basal glucose

concentration of 5.6 mM) with an additional 17 mM glucose, and a total D-glucose concentration of 22.6 mM. Many other studies from other research groups have been published using such in vitro models with concentrations of glucose in similar ranges i.e. *Vinck et al (2005) Cambier DC. Green light emitting diode irradiation enhances fibroblast growth impaired by high glucose level. Photomed laser surg. 2005; 23 (2):167-71,* and *Iannello et al (2005) Effect of in Vitro Glucose and Diabetic Hyperglycemia on Mouse Kidney Protein Synthesis: Relevance to Diabetic Microangiopathy Med Gen Med: Medscape general medicine 7(3):1*. The total glucose concentration of 22.6 mM has been added to the proceeding for further clarity, but should be read in conjunction with the cited references.

12. What was used as an osmotic control in the control/untreated cells? Were controls included at all? It is not clear from either the methodology nor the results.

Author Response: The center has published numerous studies over the years using the same models that have shown clear differences between cells grown under hyperglycemic conditions (diabetic) and cells grown under normal conditions (normal). The model used has been validated in earlier studies. The present study aimed at evaluating if PBM at 660 nm can reduce apoptosis in diabetic wounded fibroblast cells in vitro. In this case we used non irradiated cells (0 J/cm^2) as a control. A non-irradiated 'diabetic' model needs to be used as a direct control, as there are differences between 'normal' and 'diabetic' models due to the stress induced in 'diabetic'/hyperglycaemic models. PBM treated models compared to normal, unirradiated models will still show evidence of stress but this is rather due to the hyperglycemia than the effects of PBM. As compared to their own non-irradiated models, these cells show clear improvements. A sentence that reads 'Non-irradiated cells were used as controls.' Has been included.

13. The cells were seeded at a very high seeding concentration for the vessel size-how confluent were the cells at the time of treatment?

Author Response: The model used and seeding density has been validated and used and published for over 12 years. Cells are seeded to confluence; this is to enable a 'wound'/central scratch to be performed. A central scratch cannot be performed in cultures that are not confluent.

14. How many technical repeats were performed per biological repeat and how many biological repeats were included. Please provide more details regarding the use of Trypan blue to determine viability. There are several issues associated with this technique. Please refer to them as limitations in the discussion.

Author Response: Two technical repeats were performed per three biological repeats (n=3) for all experiment per time interval (0 h, 24 h and 48 h). Sample number (n=3) was included in the proceeding, and the number of technical repeats; however, the words "biological repeats" and "technical repeats" has been included for further clarity.

The authors agree that the Trypan blue exclusion assay has its limitations, as do most other viability assays such as MTT, ATP which relies on viably active cells and can be influenced if tested samples contain different cell numbers. The trypan blue assay calculates percentage viable cells based on the

number of viable and non-viable cells counted and is not influenced by metabolic activity. However, the results shown in this study show a clear correlation between increased Trypan blue and decreased caspase activity, i.e. there is increased viability and decreased apoptosis. The Trypan blue exclusion assay is a quick, cheap and reliable (with its limitations of course) assay to determine cell viability. The use of Trypan blue as a limitation to the study has been included in the proceeding.

15. How did you analyse the fluorescence images? How many images (fields of view) were taken per treatment? What microscope and objective was used? How many technical and biological repeats?

Author Response: Caspase 3/7 activity was quantitatively analysed by the Caspase Glo 3/7 assay kits, and qualitatively by immunofluorescent. Immunofluorescent images were not quantified, and the conclusions drawn are based on the quantitative analysis of caspase. To confirm a negative slide 100 fields were viewed. Up to 10 fields were viewed on a positive slide to rule out false positivity, and 2 technical slides were prepared on 3 biological repeated slides. However, according to reviewer 2 comments, the assessment of apoptosis using immunofluorescence images have been removed and have been replaced by recently obtained results on total β catenin ELISA, and 'In the activated canonical Wnt/β-catenin pathway, the cytoplasmic accumulation and nuclear translocation of β-catenin regulates arrest cell apoptosis [16]. The total β-catenin using ELISA was done to confirm the effect of PBM on apoptosis in untreated and PBM treated cells (Figure 3). There was a significant increase of total β-catenin in PBM treated DW cells ($p<0.01$) when compared to their control cells 48 h after irradiation. However, the increase observed in PBM treated D cell models was not significant when compared to their untreated D cells ($p=0.06$).' Has been added to the results section.

16. Results: Typically viability is expressed as a percentage of the untreated or vehicle control where the control is 100% viable. Why is your viability of your untreated D cells at 55%? To what did you normalize this to? Did you include untreated controls at all in your experiments? Viability assays typically also include a positive control something that will induce cell death/decrease the number of cells). Please state why you did not include a positive and negative control. To test whether there truly were any significant differences you will have to perform a 2-way anova with a relevant post-hoc test. Your 2 variables are the increased glucose concentration and treatment, and a one-way anova will not show this. You can also not compare untreated DW data to PBM-D data for example, because the conditions are completely different. Please remove the stats listed in your results section with regards to this.

Author Response: Results for Trypan blue are not normalised. An automated cell counter was used to quantify viable and non-viable cells and the percentage viability calculated. Results for % viability using Trypan blue are typically reported as non-normalised %viability. Viability in untreated diabetic cells is affected by being grown under hyperglycaemic and stressed conditions. Untreated (i.e. non-irradiated 0 J/cm^2) controls were included as shown in the graphs. When testing drugs or other compounds, positive controls can be included. This was not included in these studies.

There is one variable different between cells (all models are hyperglycaemic) control cells and irradiated cells (i.e. D0J vs D5J; and DW0J vs DW5J – the PBM/irradiation is the variable), as well as between models (i.e. D0J vs DW0J; and D5J and DW 5J – the wounding is the variable). Data for untreated DW compared to PBM-D data has however been removed.

17. For your caspase assay: what was this normalized to obtain %? What was included as positive and negative controls? Again, what did you compares this to? I.e what made up the 100%? Does the n=3 represent biological or technical repeats? In your methodology you state that only duplicates were used.

Author Response: Caspase results are not represented as a %, but rather as luminescence (reading light units) and is performed as per the manufacturer. Results for PBM treated cells (5 J/cm^2) were compared to their respective non-irradiated (0 J/cm^2) control cells. Cell culture media (no cells) plus Caspase-Glo 3/7 reagent was used to measure background luminescence and as a negative control, which was subtracted from experimental values to obtain normalised experiment values. According to the manufacturer's protocol, no positive control was included. In the methodology. 'All' has been added in the methodology to a sentence that now read as 'All experiments were repeated three times (n=3), and for the Caspase-Glo® 3/7 and β-catenin assays tests were done in duplicate, and the average of which was used.'

18. Qualitative images can be used in combination with other data to visualize observations, but no conclusions can be drawn from them. You can therefore potentially add them to you Caspase 3/7 apoptosis assay graph to show differences, but the images alone can not be shown as a result. You can only use microscopy data if you are going to quantitatively assess the fluorescence intensity and normalize this to the number of cells counted for example. This is done in software such as ImageJ and you need to assess at least 10 images per treatment with three technical repeats per biological experiment. This decrease bias (i.e picking the picture with the most or least fluorescence in it to depict what you want to see). This reviewer would therefore suggest removing these images form the manuscript.

Author Response: The authors agree, and the immunofluorescent images were never used to draw conclusions on apoptosis, but rather results obtained from the Caspase Glo assay. Plus, 10 fields of view per slide (three biological repeats) were viewed. However, based on reviewer comment and recommendation, the assessment of apoptosis using immunofluorescence images have been removed and have been replaced by total β catenin ELISA (Figure 3), and a sentence 'In the activated canonical Wnt/β-catenin pathway, the cytoplasmic accumulation and nuclear translocation of β-catenin regulates arrest cell apoptosis [16].' Has been added.

19. Conclusion: Please rewrite the 1st three sentences: 1) apoptosis is not a requirement for human development, but rather required for homeostasis, 2) what is defective apoptosis?, 3) through which

mechanisms do you think would hyperglycemia contribute to increased cellular ROS keeping in mind that increased glucose does not necessarily result in increased ATP production?

Author Response: Apoptosis is important for both physical development and homeostasis. As cells rapidly proliferate during development, some of them undergo apoptosis, which is necessary for many stages in development, including neural development, reduction in egg cells (oocytes) at birth, as well as the shaping of fingers and vestigial organs in humans and other animals (Zane Bartlett, 2017). Defective apoptosis is when cells fail to die when stimulated because of molecular abnormalities in the apoptosis pathway or in its control mechanisms. Besides its osmotic effects on cells, hyperglycaemia activates diacylglycerol (DAG) formation, together with an activation of protein kinase C and NADPH-oxidase, leading to the production of reactive oxygen species (ROS) and oxidative stress in DM.

20. Please refrain from referring to your model as diabetic. It is not. You might refer to it as hyperglycemic, but again this is not according to the published standard models for hyperglycemia. Without doing a glucose uptake assay, determining insulin resistance or evaluating GLUT4 translocation you do not know whether your model is insulin resistant which is the predecessor for diabetes.

Author Response: This is an in vitro study mimicking the in vivo diabetic environment, and have been referred to as such and published as such for many years.

21. Similarly you argue that diabetes results in or is due to oxidative stress, which in turn cause apoptosis, but you did not investigate oxidative stress. You might thus suggest that this is a potential mechanism but without investigating it you can not state that this is the case or mechanism.

Author Response: This was investigated in another study by Rajendran et al.,(2021). With limited space for this paper the investigation has been referenced.

REVIEWER #2:

1. The lack of controls in the study makes it very difficult to evaluate the robustness of the data presented.

Author Response: Controls (non-irradiated cells) were included (indicated as 0J in the graphs), and it was an oversight that this was not mentioned in the methodology. This has now been included.

2. The microscopy results are of little value as they are only qualitative. This is a pity as it is relatively simple to quantify the fluorescence yield across multiple fields of view, thereby quantifying the expression. This was not done. The scratch/wound is also not visible in the micrograph, which further reduces its value.

Author Response: The assessment of apoptosis using immunofluorescence images have been removed and have been replaced by total β catenin ELISA (Figure 3), and a sentence 'In the activated canonical Wnt/β-catenin pathway, the cytoplasmic accumulation and nuclear translocation of β-catenin regulates arrest cell apoptosis [16].' Has been added.

3. Experiments should be conducted at different seeding concentration as these high concentrations used in this study could result in the cells being completely confluent, which would greatly influence the results. This effect needs to be eliminated in the study.

Author Response: The model used and seeding density has been validated and used and published for over 12 years. Cells are seeded to confluence; this is to enable a 'wound'/central scratch to be performed. A central scratch cannot be performed in cultures that are not confluent as gaps between cells will be present.

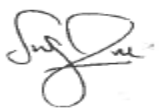
4. No proof is presented that the model used in this study is a diabetic model, which is a central aspect of the study.

Author Response: This was clearly explained in the methodology section. Diabetic cell models and the D-glucose concentrations used in this study have been tested and used for more than 12 years at Laser Research Center (LRC). The center has published studies that have shown clear differences between cells grown under hyperglycemic conditions (diabetic) and cells grown under normal conditions (normal), and this has been referenced. A hyperglycemic condition, 'Diabetic', was created by continuously growing cells in complete media (with a basal glucose concentration of 5.6 mM) with an additional 17 mM glucose, and a total D-glucose concentration of 22.6 mM. Many other studies from other research groups have been published using such in vitro models with concentrations of glucose in similar ranges.

5. In conclusion there are just too many omissions/gaps in this study to consider it for publication.

Author Response: Most of these gaps/omissions are based on a model which has been in use for over 12 years, and has been extensively published on. The model has been validated in earlier studies. The models used in this study were referenced, and due to limited space (6 pages) detailed methodology and validations cannot be included, however, having said that the models were referenced in the methodology.

Kind Regards,



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