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The action of modulated laser light on Human Very Small Embryonic-Like (hVSEL) stem cells in Platelet Rich Plasma (PRP)

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Abstract

Objective: **The biological action of a modulated low energy red end laser light has been investigated to develop an improved level of understanding regarding the potential clinical use of Human Very Small Embryonic-Like Stem Cells (hVSEL) in regenerative medicine.**

Materials and Methods: **hVSEL stem cells were confirmed to exist in the platelet compartment of the platelet rich plasma (PRP) fraction of normal peripheral blood obtained from National Health Service Blood and Transfusion (NHSBT).**

Results: **hVSEL stem cell proliferation was identified in PRP using flow cytometry for the surface antigens known to be expressed by hVSEL stem cells (Oct 3/4, SSEA4 and CXCR4) in the lineage negative (Lin-) compartment. The experiments confirm the presence of hVSEL stem cells in PRP which were then exposed to a SONG modulated 5 mW, 670 nm red laser light, adjusted through optical phase conjugation to 1 mW output for 3 minutes and with variations in modulation and laser exposure times. The resultant laser exposed hVSEL stem cells were then re-assessed for cell proliferation using flow cytometry. Those hVSEL stem cells exposed to laser light were shown to have an increase in hVSEL stem cell proliferation compared to controls.**

Conclusions: **This is the first report of hVSEL stem cell proliferation in response to modulated laser light.**

Introduction

hVSEL Stem Cells

In the normal physiological state stem cells are continuously in transit to and from the bone marrow *via* the peripheral blood and lymphatic system[1-3]. hVSEL stem cells have been shown to be present in human umbilical cord blood[4] and are described as small (1-4 μm) non-haemopoietic cells with a high nuclear/cytoplasm ratio. They express similar surface antigens to pluripotent embryonic stem cells. hVSEL stem cells in adult bone marrow have been shown to be CXCR4+, CD34+, CD133+, Oct4+, SSEA4+ and lin-, CD45-[5]. hVSEL stem cells were subsequently confirmed to be present in peripheral blood and bone marrow and in leucopheresis samples taken following G-CSF administration[6]. hVSEL stem cells have since been described by many workers in the peripheral blood at a concentration of 800-1300 cells/mL[7].

It has been proposed that hVSEL stem cells are a population of epiblast-derived cells created during embryonic gastrulation. In addition, it is postulated that hVSEL stem cells may be important in the long-term production of CD34+ haemopoietic stem cells in the bone marrow[8]. More recently it has been shown that hVSEL stem cells can contribute to repair in experimental myocardial infarction (MI) which, if translated to a clinical protocol in the future, could make a significant new treatment modality in MI using hVSEL stem cells[9].

It has also recently been shown that hVSEL stem cell numbers in peripheral blood persist throughout life[10]. This is a very important observation because if hVSEL stem cells can be translated to clinical use then it may be possible to obtain autologous hVSEL stem cells from any patient at any age. This would

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be a considerable advantage in the use of stem cells in regenerative medicine, simplify procedures, save money and reduce any possible adverse reactions associated with allogeneic cells.

Other workers are proposing that hVSEL stem cells are a better option to potentially develop pancreatic tissue and human gametes rather that iPSC and hESC and it is argued that the ontogeny of hVSEL stem cells is the reason behind the preferred utility of hVSEL stem cells in this context[11].

These observations of hVSEL stem cells suggest that, with the correct handling and administration, they could play a critical part in translational regenerative medicine in the future.

Laser and Light Technology in Stem Cell Biology

There is a considerable amount of interest and discussion at present about the potential role of laser and light technology in stem cell biology.

Stem cells isolated from human exfoliated deciduous teeth (SHED) have been exposed to laser light (660 nm, 30 mW) in order to assess the effect of the laser on stem cell proliferation. The conclusion here was that laser exposure does promote proliferation of SHED stem cells and maintained viability when compared to control cells[12]. Similar work has been carried out using laser radiation at 420 nm, 540 nm, 660 nm, and 810 nm to assess the effect of varying laser wavelength on human adipose tissue derived stem cells. The wavelength was varied but the power of the laser remained the same at 3 J/cm^2. It was found that the 420 nm and 540 nm laser wavelengths stimulated osteogenic differentiation whereas the other wavelengths did not. The authors hypothesise that the laser could be activating light-gated calcium ion channels[13,14].

Other workers have written about the 'photobiomodulation' of stem cells using visible and infrared light and found that increased power density and reduced energy density of the light source optimises the effect of light on stem cells[15]. More recently photobiomodulation, along with the use of nutraceuticals, has been shown to be a possible treatment for dry age-related macular degeneration[16]. Broadband visible light (low-level visible light) has been shown to increase proliferation of bone marrow MSC *in vitro* and this has raised the suggestion that such an approach may be helpful in wound healing[17].

It has also been shown that exposure of rat diabetic MSC to a helium-neon laser at 632.8 nm resulted in improved cell survival, proliferation and apoptosis. The authors suggest that photobiomodulation could be a useful intervention for MSC *in vitro* and also for MSC *in vivo* to enable the cells to proliferate in their niche[18].

Many other authors have described the photobiomodulation effects of laser light on dental pulp MSC, human adipose MSC and epithelial colony forming units[19-21]. In this work we assess the modulated laser photobiomodulation effects on the proliferation of peripheral blood hVSEL stem cells in PRP.

Materials and Methods

Background Light

All processing and manipulations of human PRP containing hVSEL stem cells in these experiments were carried out in minimum background white light conditions to minimise the possible effects of natural or generated white light on the hVSEL stem cells in PRP.

Donated Human Peripheral Blood

Anti-coagulated (sodium citrate) donated normal human peripheral blood (450 mL) was purchased from the National Health Service Blood and Transfusion (NHSBT) and kept at 4°C before use. The blood was allowed to warm to room temperature before processing for PRP.

Preparation of Platelet Rich Plasma (PRP) Containing hVSEL Stem Cells

All preparations of PRP from human peripheral blood and manipulation of cultured PRP were carried out in a Grade B clean room using aseptic technique.

PRP for all of the studies was prepared from donated human peripheral blood as described below. Each sample of PRP was obtained by using 3x11mL PRP tubes (Qigeneration – Quantum information for regeneration) into which 11 mL of whole peripheral blood was added. The whole blood PRP tubes were centrifuged (Qigeneration) at the pre-set g force for 10 minutes. Each of the three 11 mL PRP tubes produced approximately 6 mL (a total of approximately 18 mL) of PRP which was aliquoted, using aseptic technique in a Class II flow hood, into a single sterile tube for further manipulation and analysis. Each 18mL PRP preparation was created in triplicate for each manipulation and assessment process.

Flow Cytometry

All PRP samples were analysed using a MACSQuant (Miltenyi Biotec, Bergisch Gladbach, Germany) flow cytometer utilising fluoresence-1 to set the appropriate channels, voltages and controls for hVSEL stem cell analysis.

All flow cytometry was controlled by counting a PRP sample with no antibodies added. The CXCR4, Oct3/4 and SSEA counts in these controls were always zero. A typical flow cytometer plot for untreated PRP is shown in Figure 1.

Each sample for all hVSEL stem cell analysis was assessed in triplicate.

All PRP samples were diluted in saline 1:10 (v/v) prior to staining with the following reagents:

- Lineage Cell Depletion Kit (Miltenyi Biotec) (130-092-211).
- Mouse anti-human CD184(CXCR4)-PE (Thermo-Fisher Scientific, Waltham, MA, USA) (12-9999-42).
- Mouse anti-human CD34-PE (Miltenyi Biotec) (130-120-515).
- Mouse anti-human CD133-APC (Miltenyi Biotec) (130-113-668).
- Recombinant human IgG1 CD45-APC VIO 770 (Miltenyi Biotec) (130-110-773).
- Rat anti-humanOCT3/4 PE (ThermoFisher Scientific) (12-5841-82).
- Mouse anti-human SSEA-1 Alexa Flour 488 (ThermoFisher Scientific) (53-8813-42).
- Recombinant human Biotin antibody Vio-Bright 515 (Miltenyi Biotec) (130-111-074).
- Propidium Iodide (PI) for viable cell exclusion (Miltenyi Biotec) (130-093-233).
- All reagents were used following the manufacturers' instructions.

Lasers and SONG Laser Modulation

Two lasers were used in this study:

- Costa Laser: this was a 670 nm 5 mW (Sanyo, Osaka, Japan) SONG modulated laser with the SONG set at 60% optical phase conjugation (OPC) for a resultant beam power of 1 mW unless this level of optical phase conjugation was varied for experimental purposes.
- Magna Costa Laser: this was a 670 nm 5.7 mW (Power Technology) SONG modulated laser with the SONG set at 60% OPC for a resultant

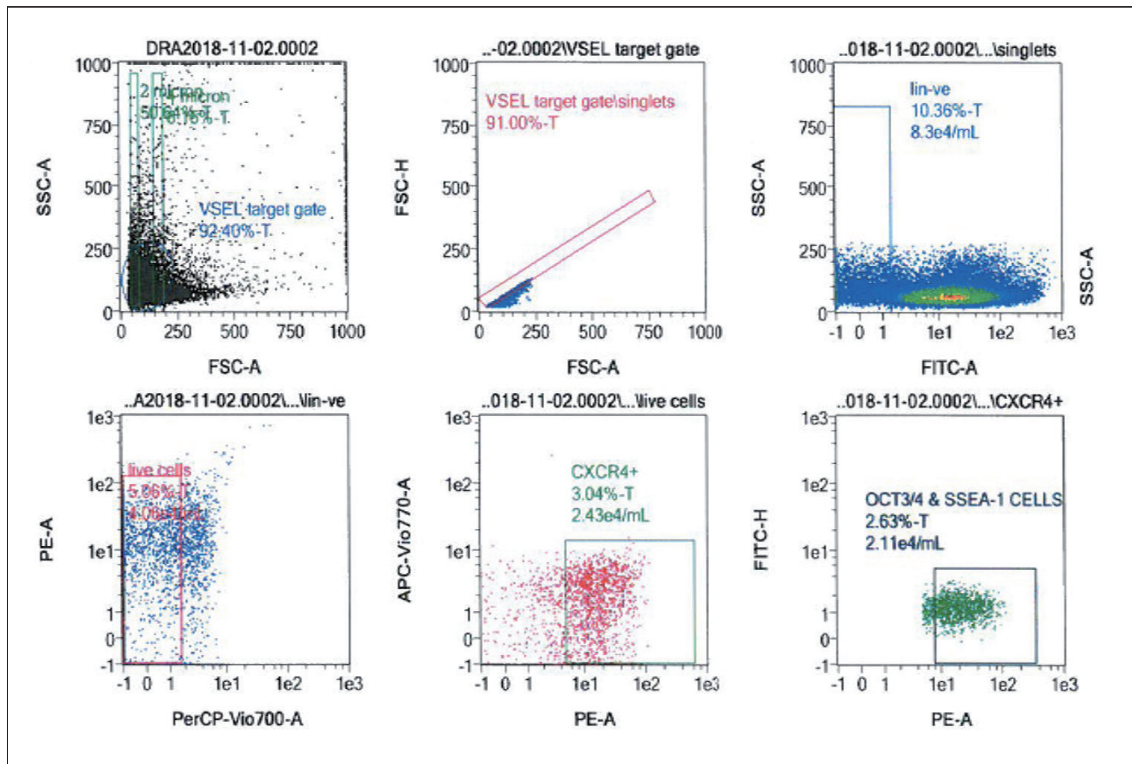


Figure 1. A typical flow cytometer result for PRP with no treatment.

beam power of 1.3 mW. The Magna Costa laser has adjustable wave forms to enable alternative wave forms to be introduced as a control.

SONG Laser Modulation

SONG stands for Strachan-Ovokaitys Node Generator and was developed by the third author of this paper in collaboration with his physicist colleague Scott Strachan.

SONG modulation of the laser cancels the central wavelength band of the laser output in a process described as non-fringing destructive interference. The remaining upper and lower wavelength bands create a beat frequency pattern of sparse nodes of constructive interference which represents the physical visible light that remains.

Modulation of this complex wave form pattern results in a rapid traverse of these nodes that can reach pulse repetition frequencies at intervals as rapid as subfemtosecond. The destructive interference and sparseness of the nodes reduces the flare at the surface of the tissue interface. This decreases both the reflectiveness of photons which have entered a zone that has just experienced a photon absorption as well as the related scattering effect. The depth of penetration of sparse nodes may be 10-20 times that of ordinary photons at the surface of an interface such as human skin.

Culture and Harvesting of Laser Treated and Control (No Laser or White Light) hVSEL in PRP

Where culture of PRP was needed to assess the biological stability of the effect of laser exposure the PRP was cultured in equal volumes of RPMI 1640 media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 200 mM L-Glutamine (Sigma-Aldrich), penicillin and streptomycin (Sigma-Aldrich) and 10% heat inactivated fetal calf serum (FCS; Thermo-Fisher).

All PRP cultures were carried out using T25 vented flasks (Thermo-Fisher) in a humified incubator (Thermo-Fisher) set at 37°C and 5% CO_2 in air.

Adherent cells were harvested when needed by in an initial wash with Ca^{2+}/Mg^{2+} free Dulbecco's PBS (Thermo-Fisher) and treatment with Trypsin EDTA (Thermo-Fisher) for 5 minutes at 37°C.

The Numbers and Distribution of hVSEL Stem Cells in Untreated PRP

In order to assess the distribution and numbers of hVSEL stem cells in untreated PRP each of the triplicate PRP tubes were sampled into separate tubes following centrifugation by taking 2 mL of the 'top' PRP, 2 mL of the 'middle' PRP, 2 mL of the 'bottom' PRP close to the red cell interface as possible, 2 mL of the top of the red cell section and 2 mL at the bottom of the red cell section. Each sample was assessed for hVSEL stem cell numbers using the flow cytometry protocol above.

Laser Treatment (Costa) of PRP and Resulting hVSEL Stem Cell Proliferation on Day 0 and Day 1 of Culture

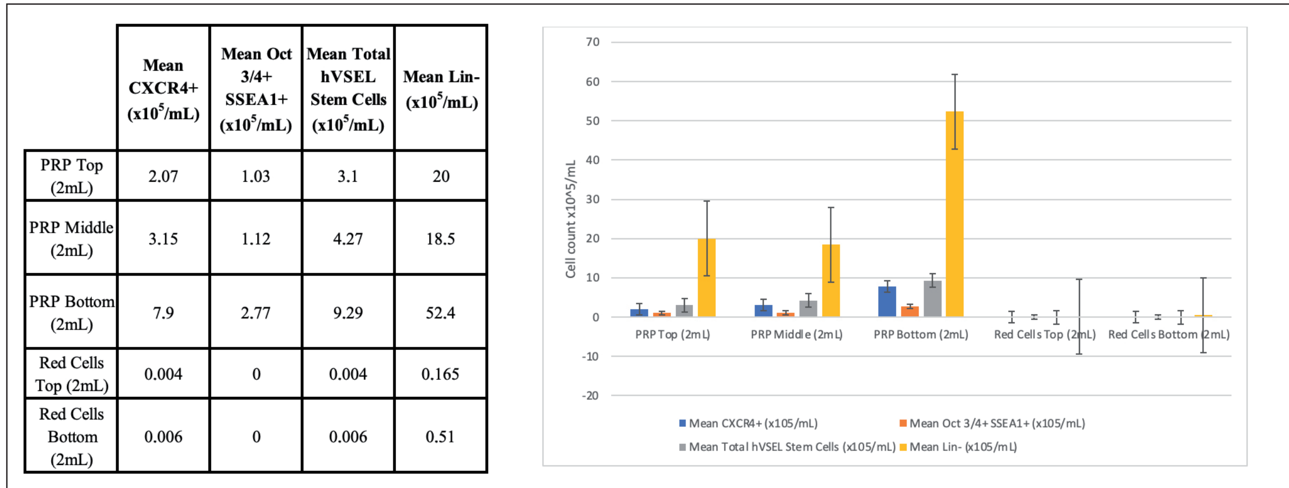
This was the initial experiment to assess the effect of the Costa laser with SONG modulation on hVSEL stem cell numbers in PRP. PRP was prepared as above, in triplicate, and one batch was exposed to Costa laser +SONG (set at 60% OPC) light for 3 minutes, one batch was exposed to white torch light for 3 minutes, and one batch received no treatment (control). Following flow cytometer analysis, the 3 PRP samples were cultured as described as above and then harvested as described above for flow cytometry analysis on day 1.

Laser Treatment (Magna Costa) of hVSEL Stem Cells in PRP With Titration of Laser Exposure Time and ±SONG Modulation at D0 and D5

The purpose of the experiment was to assess the numbers of hVSEL present in PRP following laser exposure from 1-3 minutes with and without the SONG modulation. This will confirm optimum settings for clinical use. The Magna Costa laser is the same as the Costa except for an adjustable wave form. This enables the use of a possibly improved control of a 'flat' wave in these experiments. The SONG modulation was set at 60% OPC throughout the experiment. All cells were analysed at Day 0 and then cultured *in vitro* for 5 days to assess the persistence of any proliferative changes in hVSEL. Culture and harvesting protocols were as described above.

Costa Laser Treatment (± SONG Modulation) of hVSEL stem cells in PRP at Day 0, Day 1 and Day 7

PRP was prepared as above and the PRP exposed to the Costa laser for 3 minutes with and 3 minutes without SONG modulation. The resultant PRP was then assessed for hVSEL proliferation



	Mean CXCR4+ ($x10^5$/mL)	Mean Oct 3/4+ SSEA1+ ($x10^5$/mL)	Mean Total hVSEL Stem Cells ($x10^5$/mL)	Mean Lin- ($x10^5$/mL)
PRP Top (2mL)	2.07	1.03	3.1	20
PRP Middle (2mL)	3.15	1.12	4.27	18.5
PRP Bottom (2mL)	7.9	2.77	9.29	52.4
Red Cells Top (2mL)	0.004	0	0.004	0.165
Red Cells Bottom (2mL)	0.006	0	0.006	0.51

Figure 2. Gradient of hVSEL stem cells in peripheral blood PRP.

and then put into *in vitro* culture as described above for 1 and 7 days. Cultures were harvested on Day 1 and Day 7 as described above and the resultant cell harvest was the assessed for hVSEL proliferation using flow cytometry. This experiment also included an assessment of hVSEL numbers in whole peripheral blood following red cell lysis.

Time Titration of SONG modulated Magna Costa and Costa Laser on hVSEL Stem Cells in PRP

PRP was prepared as above and exposed to the Magna Costa laser for 3 minutes and the Costa laser for 3,6 and 9 minutes. White light and no treatment controls were used. hVSEL stem cell flow cytometer analysis was carried out for all exposure times.

Results

The numbers and PRP distribution of hVSEL stem cells in untreated PRP

Cell viability remained at >90% in all experiments. The data on the numbers and distribution of hVSEL stem cells in untreated PRP are summarised in Figure 1. A typical flow cytometer result for untreated PRP is shown in Figure 1.

The top 2 mL of the PRP was found to have a mean hVSEL stem cell count of 3.1 $x10^5$/mL and the mean Lin- cell count was 20.0 $x10^5$/mL.

The middle 2 mL of the PRP was found to have a mean hVSEL stem cell count of 4.27 $x10^5$/mL and the mean Lin- cell count was 18.5 $x10^5$/mL.

The bottom 2 mL of the PRP was found to have a mean hVSEL stem cell count of 9.29 $x10^5$/mL and the mean Lin- cell count was 52.2 $x10^5$/mL.

The total mean number of hVSEL stem cells found in PRP was 1.66 $x10^6$/mL.

The total mean number of Lin- cells found in the PRP was 9.01 $x10^6$/mL.

The total number of hVSEL stem cells in the red cell top section was 4.0 $x10^2$/mL and the mean Lin- cell count was 1.65 $x10^4$/mL.

The total number of hVSEL stem cells in the red cell bottom section was 6.0 $x10^2$/mL and the mean Lin- cell count was 5.1 $x10^4$/mL (Figure 2).

Laser Treatment of PRP and Resulting hVSEL Stem Cell Numbers on Day 0 and Day 1 of Culture

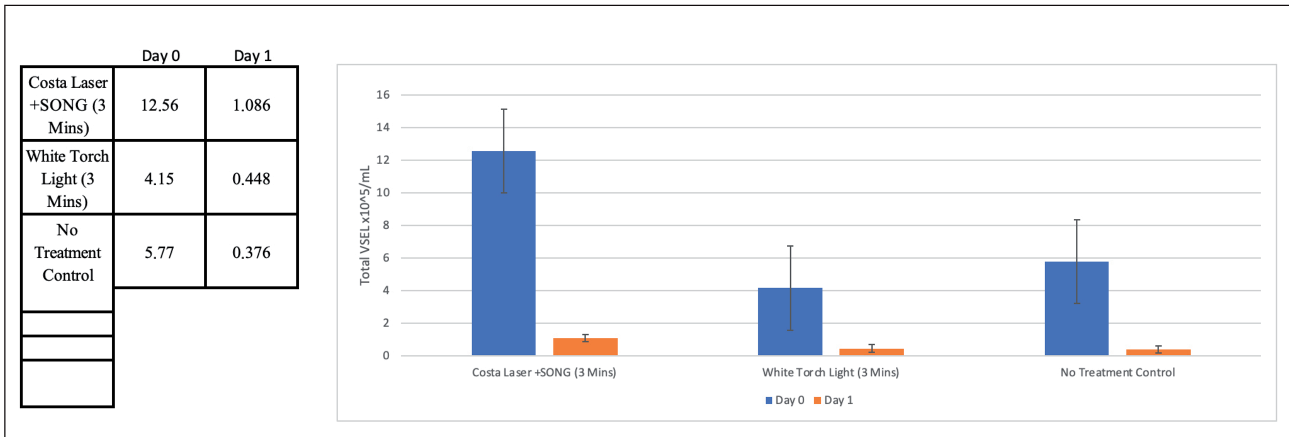
The data on Costa laser +SONG modulation of PRP, related controls and *in vitro* culture for one day are summarised in Figure 3.

When PRP was treated with laser light +SONG for 3 minutes, and analysed by flow cytometry immediately afterwards, the number of hVSEL stem cells were 1.256 $x10^6$/mL.

The same batch of PRP treated with white torch light for 3 minutes and analysed immediately contained 4.15 $x10^5$/mL hVSEL stem cells.

The same batch of PRP, undergoing no treatment as a control, contained 5.77 $x10^5$/mL hVSEL stem cells.

On day 1 of culture *in vitro* (the cells were harvested as described above) the Costa laser +SONG PRP contained 1.086 $x10^5$/mL hVSEL stem cells.



	Day 0	Day 1
Costa Laser +SONG (3 Mins)	12.56	1.086
White Torch Light (3 Mins)	4.15	0.448
No Treatment Control	5.77	0.376

Figure 3. Laser Treatment of PRP and Resulting hVSEL Stem Cell Proliferation on Day 0 and Day 1 of Culture.

On day 1 of culture *in vitro* (the cells were harvested as described above) the White Torch light PRP contained 0.448 x10^5/mL hVSEL stem cells.

On day 1 of culture *in vitro* (the cells were harvested as described above) the Control PRP (no treatment) contained 0.376 x10^5/mL hVSEL stem cells.

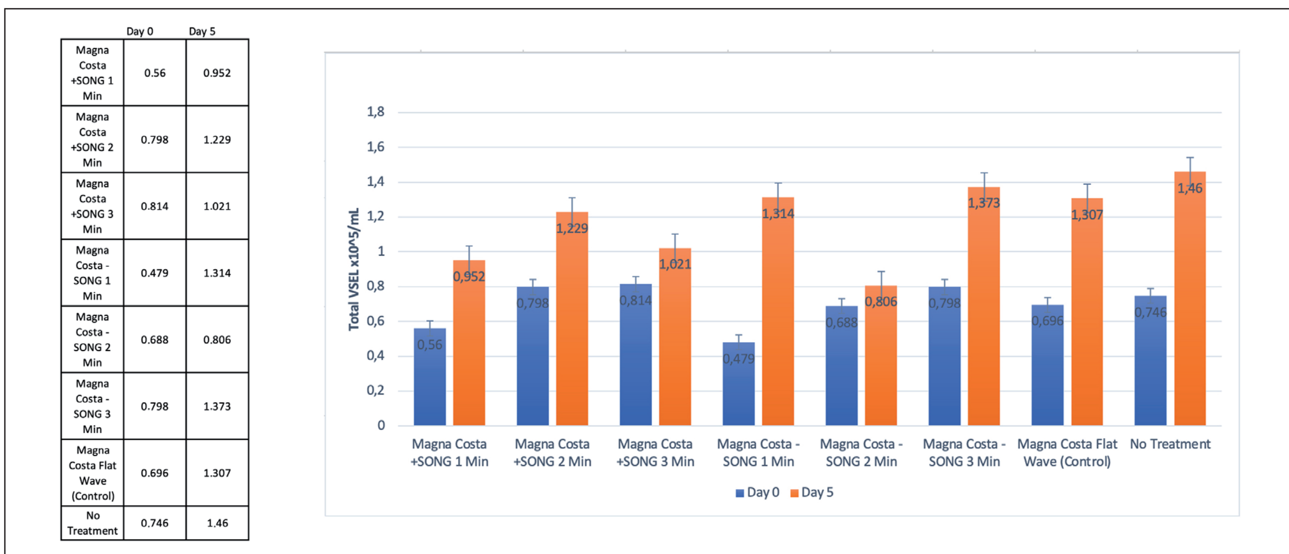
Magna Costa Laser Exposure Time Variation and SONG Modulation Variation on Day 0 and Day 5

Fresh Day 0 ± SONG Modulation 1-3 Minute Laser Exposure

On day 0 (the day when the PRP was prepared and lasered) the total number of hVSEL stem cells in the PRP increased as the laser exposure time was increased (from 1 minute to three minutes) and the SONG modulation was present throughout. The 2 and 3 minutes laser exposure time produced very similar numbers of hVSEL stem cells. There was a similar but less pronounced rise in hVSEL stem cell numbers when the laser was applied without SONG modulation. The flat wave and no treatment controls remained similar and the flat wave laser exposure time here was 3 minutes (Figure 4).

In Vitro *Day 5± SONG Modulation 1-3 Minute Laser Exposure*

On day 5 of culture *in vitro* the SONG modulated laser group showed increased numbers of hVSEL stem cells compared to Day 0 with slightly more hVSEL stem cells present in the 2 and 3 minute laser expo-



	Day 0	Day 5
Magna Costa +SONG 1 Min	0.56	0.952
Magna Costa +SONG 2 Min	0.798	1.229
Magna Costa +SONG 3 Min	0.814	1.021
Magna Costa - SONG 1 Min	0.479	1.314
Magna Costa - SONG 2 Min	0.688	0.806
Magna Costa - SONG 3 Min	0.798	1.373
Magna Costa Flat Wave (Control)	0.696	1.307
No Treatment	0.746	1.46

Figure 4. Magna Costa Laser Exposure Time Variation and SONG Modulation Variation on Day 0 and Day 5.

sure time. The 1 and 3 minute laser exposure without SONG modulation contained more hVSEL stem cells than the 2 minute laser exposure and the flat wave and no treatment controls also contained more hVSEL stem cells overall than in Day 0 (Figure 4).

Costa Laser Treatment (± SONG Modulation) of hVSEL Stem Cells in PRP at Day 0, Day 1 and Day 7

This experiment was designed to assess the numbers of hVSEL stem cells in PRP on the day of laser treatment and at Day 1 and Day 7 culture of the cells *in vitro* and to assess the effect of laser treatment with and without SONG modulation. This is to try to understand the behaviour of laser treated hVSEL stem cells *in vitro* which may give an insight into the behaviour of hVSEL stem cells *in vivo* and also the importance of SONG modulation. The results can be seen in Figure 5.

A measurement was made on the number of hVSEL stem cells in peripheral blood without any treatment. This involved red cell lysis followed by flow cytometry. The number of hVSEL stem cells in this sample of peripheral blood was $8.1x10^5$/mL which correlates well with previous estimates of hVSEL stem cells in PRP at $1x10^6$/mL and the hVSEL stem cells in PRP control in this experiment of $1.072x10^6$/mL. It is to be expected that PRP will have slightly higher hVSEL stem cell counts than peripheral blood as hVSEL stem cells are concentrated in PRP. The number of hVSEL stem cells in PRP following 3 minutes of SONG modulated laser treatment was increased to $2.22x10^6$/mL, on Day 1 of culture it was $7.82x10^5$/mL and on day 7 of culture it was $2.56x10^5$/mL. The number of hVSEL stem cells in PRP following 3 minutes of unmodulated laser treatment was increased to $1.994x10^6$/mL, on Day 1 of culture it was 1.348 $x10^6$/mL and on day 7 of culture it was 1.48 $x10^5$/mL.

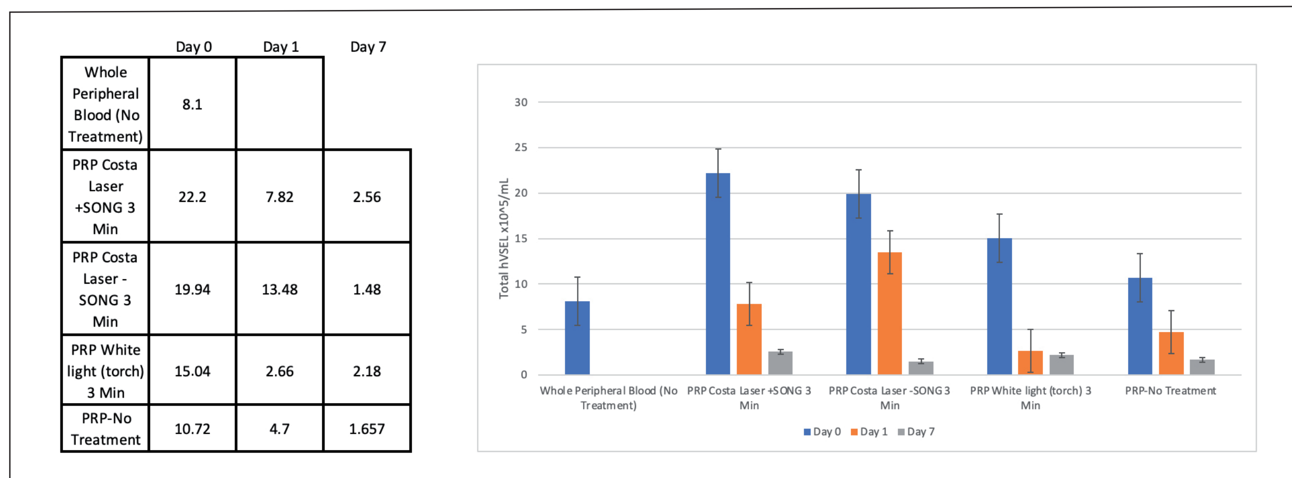
The number of hVSEL stem cells in PRP following 3 minutes of white light treatment (as a presumed control) was increased to 1.504 $x10^6$/mL, on Day 1 of culture it was 2.66 $x10^5$/mL and on day 7 of culture it was 2.18 $x10^5$/mL.

The number of hVSEL stem cells in PRP following no treatment (as a control) was 1.072 $x10^6$/mL, on Day 1 of culture it was 4.7 $x10^5$/mL and on day 7 of culture it was 1.657 $x10^5$/mL.

Time Titration of SONG modulated Magna Costa and Costa Laser on hVSEL Stem Cells in PRP

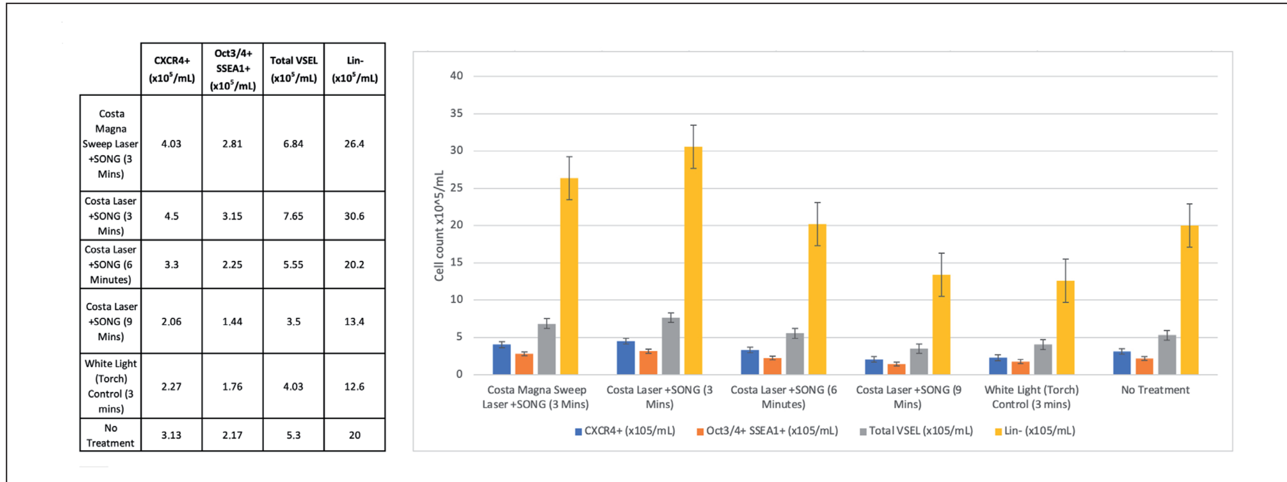
The purpose of this experiment was to identify the optimum laser exposure time for the proliferation of hVSEL stem cells in PRP. The results can be seen in Figure 6.

The total hVSEL stem cells found in PRP exposed to the SONG modulated Costa Magna and the Costa laser for three minutes were higher than exposure to the SONG modulated Costa laser for 6 or 9 minutes. These data confirm that the optimum laser exposure time to maximise hVSEL stem cell proliferation is 3 minutes. The white light (torch) control and the no treatment control showed hVSEL stem cell numbers less than the 3 minute SONG modulated laser exposure confirming the optimised exposure time to 3 minutes.



	Day 0	Day 1	Day 7
Whole Peripheral Blood (No Treatment)	8.1		
PRP Costa Laser +SONG 3 Min	22.2	7.82	2.56
PRP Costa Laser -SONG 3 Min	19.94	13.48	1.48
PRP White light (torch) 3 Min	15.04	2.66	2.18
PRP-No Treatment	10.72	4.7	1.657

Figure 5. Costa Laser Treatment of hVSEL stem cells in PRP at Day 0, Day 1 and Day 7.



	CXCR4+ (x10^5/mL)	Oct3/4+ SSEA1+ (x10^5/mL)	Total VSEL (x10^5/mL)	Lin- (x10^5/mL)
Costa Magna Sweep Laser +SONG (3 Mins)	4.03	2.81	6.84	26.4
Costa Laser +SONG (3 Mins)	4.5	3.15	7.65	30.6
Costa Laser +SONG (6 Minutes)	3.3	2.25	5.55	20.2
Costa Laser +SONG (9 Mins)	2.06	1.44	3.5	13.4
White Light (Torch) Control (3 mins)	2.27	1.76	4.03	12.6
No Treatment	3.13	2.17	5.3	20

Figure 6. Time titration of Magna Costa sweep and Costa laser on hVSEL stem cells in PRP.

Discussion

The purpose of this set of experiments was to explore the possible biological effects of a low energy (1 mW) SONG modulated red laser (670 nm) on the proliferation of hVSEL stem cells in PRP. These observations may provide an initial basis and understanding of the encouraging unpublished data seen in the clinical use of laser treated hVSEL stem cells in PRP in a Phase I clinical trial for heart failure and in treatment of neurodegenerative disease (Ovokaitys *et al.*, in preparation).

The Numbers and Distribution of hVSEL stem cells in untreated PRP

The quantification of hVSEL stem cells in whole blood, in PRP and in other tissues has been reported many times[22,23]. There is a wide range of hVSEL stem cell count depending on the source of blood or tissue and the flow cytometery protocol used. The same authors report possible mobilisation of hVSEL stem cells in disease and more recently workers have suggested that hVSEL stem cells may also be involved in oncogenesis[24].

In the present study we showed that there was a mean of 1.6 x10^6/mL hVSEL stem cells in PRP obtained from donated human blood. It was also possible to provide another mean estimate of the total hVSEL stem cells/mL in PRP by taking the mean of the no treatment values for PRP across all of the different experiments. This gives a mean value of 3.92x10^6/mL. The range of observed hVSEL stem cells in PRP normal peripheral blood in the present gradient study was 0.746-16 x10^5/mL. The PRP was found to have a gradient of hVSEL stem cells increasing from the top meniscus of the PRP all the way down to the PRP/red cell interface where the highest number of hVSEL stem cells were found. This confirms that the whole 6mL volume of PRP should be used for optimal results. Multiple 6mL volumes of PRP are pooled for clinical applications. There were very few (approximately 1x10^3/mL) hVSEL stem cells in the red blood cell section of the PRP tube. These data show that the PRP based isolation of hVSEL stem cells works very efficiently when using the technology described. hVSEL stem cell viability remained high throughout all experiments.

Laser Treatment of PRP and Resulting hVSEL Stem Cell Proliferation on Day 0 and Day 1 of Culture

The purpose of this experiment was to assess the initial effects of the laser on hVSEL stem proliferation and to see if these changes were stable after 24 hours in culture *in vitro*. Other workers have described gene upregulation in human dermal cells following laser exposure which resulted in increased paracrine secretions[25].

The total hVSEL stem cells in the PRP following 3 minutes of SONG modulated laser exposure was 1.256x10^6/mL. The white light (torch control) PRP had a total of 4.15x10^5/mL hVSEL stem cells and the no treatment control had 5.77x10^5/mL hVSEL stem cells. The mean of these 2 control samples was 4.96x10^5/mL. The laser exposed PRP therefore showed a 2.5 times increase in hVSEL stem cell num-

bers compared to the mean of the two control groups. This was a rapid effect in that following modulated laser exposure the cells were taken immediately for analysis on the flow cytometer. The time from modulated laser exposure to flow cytometry analysis was, therefore, no greater than 30 minutes in any of the experiments. This observation compares favourably with the clinical use and clinical trial of modulated laser exposed hVSEL in PRP which often show rapid clinical improvements following intravenous infusion of autologous laser exposed hVSEL stem cells in PRP. This is the first time that these laboratory observations and clinical data have been correlated.

Following 24 hours culture *in vitro* the modulated laser exposed hVSEL were 1.086x10^5/mL compared to 0.448x10^5/mL in the white light torch control and 0.376x10^5/mL in the no treatment control. The mean of these two control groups is 0.432x10^5/mL. The laser exposed PRP after 24 hours *in vitro* showed a 2.5 times increase of hVSEL stem cells over the control cells indicating that even though the actual cell counts decreased (which is to be expected following culture *in vitro*) the ratio of laser modulated hVSEL stem cells to control hVSEL stem cells remained the same over 24 hours.

These data have confirmed, to our knowledge for the first time, that the SONG modulated laser has a proliferative effect on human hVSEL stem cells in PRP. This effect was maintained in relative terms for at least 24 hours *in vitro* post laser exposure. Further work is needed on the possible paracrine, surface antigen un-masking and gene upregulation effects of laser treatment of hVSEL stem cells.

Magna Costa Laser Exposure Time Variation and SONG Modulation Variation on Day 0 and Day 5

The purpose of this experiment was to assess the laser exposure time and the application of SONG modulation, or no SONG modulation, on the proliferation of hVSEL stem cells in PRP on the day of laser exposure (D0) and after five days *in vitro* (D5). The laser exposure times and SONG modulation are thought to be critical to successful hVSEL stem cell proliferation.

Day 0

In the PRP exposed to the SONG modulated Magna Costa laser for 1,2 and 3 minutes the numbers of hVSEL stem cells were highest in the 2 and 3 minute treatments. In the Magna Costa laser without SONG modulation there were fewer hVSEL stem cells than in the laser SONG modulated group over 1,2 and 3 minutes but there was a steady increase in detected hVSEL stem cells across the laser exposure times. The SONG modulated Magna Costa flat wave and no treatment controls (hVSEL numbers) were lower than the equivalent SONG modulated laser cell counts at 2 and 3 minute laser exposure.

Day 5

The Day 5 hVSEL stem cell counts, after 5 days culture *in vitro*, all showed an increase in hVSEL stem cells compared to Day 0. There was also an increase in the control groups which appeared greater than the experimental groups. This anomaly needs further investigation because it could be a true reflection of *in vitro* proliferation of hVSEL stem cells or it may just be an anomaly in this particular experiment. In general terms when lasered hVSEL stem cells were cultured *in vitro* in the other experiments in this paper then a reduction in cell numbers was observed.

Costa Laser Treatment of hVSEL stem cells in PRP at Day 0, Day 1 and Day 7

Day 0

This experiment confirmed the presence of hVSEL stem cells in whole peripheral blood after red cell lysis. These are important data and the PRP data all showed an increase in hVSEL stem cell numbers in PRP which helps to confirm that PRP is an efficient route to isolate hVSEL stem cells for experimental and clinical use.

The highest numbers of hVSEL stem cells in PRP were found in the Costa laser with SONG modulation with a 3 minute exposure time. The same laser exposure without SONG modulation showed fewer hVSEL stem cells but still increased levels over controls indicating some possible benefits of laser exposure even without SONG modulation. The white light and no treatment controls both showed fewer hVSEL stem cells than the SONG modulated and SONG unmodulated treatments.

Day 1 and Day 7

The numbers of hVSEL stem cells present after 1 and 7 days of culture *in vitro* decreased which may reflect cell death related to *in vitro* culture.

Time titration of Magna Costa and Costa laser on hVSEL stem cells in PRP

These experiments were to assess the extended laser exposure time on the proliferation of hVSEL in PRP.

The 3 minute laser exposure provided more hVSEL stem cell proliferation than the 6 or 9 minute exposure. The confirms the optimum laser exposure time for hVSEL stem cell proliferation is 3 minutes.

Conclusions

It is clear from the present data and the corresponding unpublished clinical data that laser activation of hVSEL stem cells in PRP results in hVSEL stem cell proliferation. This has a great potential in future routine therapy and also in understanding the true nature of hVSEL stem cells. There are many things which still need to be done and these include:

- An assessment of hVSEL stem cells in freshly collected normal blood, freshly collected blood from prospective patients and freshly collected hVSEL stem cells in freshly collected cord blood. The research in this paper used donated adult blood which may not be giving the true hVSEL stem cell numbers which may be seen in fresh normal and fresh patient blood.
- Optimisation of PRP preparation for laser activation of hVSEL stem cells.
- Optimisation of the laser modulation for best effects. This will be a long-term project as we expect ongoing R&D in this field.
- Gene activation and regulation studies are needed to understand the genetic mechanism of laser induced hVSEL stem cell proliferation.
- The immediate post-laser treatment paracrine secretions of laser activated hVSEL stem cells needs analysis. This may be important in the overall efficacy of laser activated hVSEL stem cells *in vivo.*
- Investigation of possible 'un-masking' of hVSEL stem cell surface antigens (e.g. CXCR4) following modulated laser treatment.
- The optimisation of hVSEL collection to perhaps exclude platelets for further clarity.
- The possibility of freezing hVSEL stem cells to create donor banks for future autologous and allogeneic work.
- The possibility of *in vitro* expansion of hVSEL stem cells to enable to assessment of dose response using laser activated hVSEL stem cells.

In addition to the work above we are also considering possible quantum biology explanations of the observed effects of modulated laser light on hVSEL stem cells. Quantum biology has already been used to describe photosynthesis, olfaction and navigation by some birds[26]. This level of understanding will bring the hope of a better understanding of disease and a solid foundation for future stem cell technologies using interventions such as modulated laser light.

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Peter Hollands: Composing and revising manuscript, experimental design, data acquisition analysis and interpretation and joint final approval of manuscript for publication. Damilola Oboyeji: Experimental design, data acquisition and manuscript review. Todd Ovokaitys: Funding of the work, experimental design, data analysis, manuscript revision and joint final approval of manuscript.

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Conflict of Interest:

Dr Ovokaitys is CEO of Qigenix. Prof Hollands and Mr Obeyeji have no conflict of interest to disclose.

References

1. LaLevesque JP, Winkler IG, Larsen SR, Rasko JEJ. Mobilization of bone marrow-derived progenitors in Bone Marrow-Derived Progenitors, 180 Handbook of Experimental Pharmacology. Springer, Berlin, Germany 2017; pp. 3-36.
2. Quesenberry PJ, Colvin G, Dooner G, Dooner M, Aliotta JM Johnson K. The stem cell continuum: cell cycle, injury, and phenotype lability. Ann New York Acad Sci 2007; 1106: 20-29.
3. Massberg S, von Andrian UH. Novel trafficking routes for hematopoietic stem and progenitor cells. Ann New York Acad Sci 2009; 1176: 87-93.
4. Kucia M, Halasa M, Wysoczynski W. Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ Oct-4+ very small embryonic-like cells purified from human cord blood—preliminary report. Leukemia 2007; 21: 297-303.
5. Kucia M, Reca R, Campbell FR. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 2006; 20: 857-869.
6. Sovalat H, Scrofani M, Eidenschenk A. Identification and isolation from either adult human bone marrow or G-CSF-mobilized peripheral blood of CD34+/CD133+/

CXCR4+/ Lin−CD45− cells, featuring morphological, molecular, and phenotypic characteristics of very small embryonic-like (VSEL) stem cells. Exp Hemat 2011; 39: 495-505.

7. Wojakowski W, Kucia M, Liu R, Zuba-Surma, EK, Jadczyk T, Bachowski R, Nabialek E, Kazmierski M, Ratajczak MZ, Tenera, M. Circulating very small embryonic-like stem cells in cardiovascular disease. J Cardiovasc Trans Res 2011; 4: 138-144.
8. Ratajczak MZ, Zuba-Surma EK, Ratajczak J, Wysoczynski M, Kucia M. Very Small Embryonic Like (VSEL) Stem Cells – Characterization, Developmental Origin and Biological Significance. Exp Hematol 2008; 36: 742-751.
9. Wojakowski W, Kucia M, Zuba-Surma, E, Jadczyk T, Ksiazek B, Ratajczak MZ, Tendera M. Very Small Embryonic-Like Stem Cells in Cardiovascular Repair. Pharmacol Ther 2011; 129: 21-28.
10. Sovalat H, Scrofani M, Eidenschenk A, Hénon P. Human very small embryonic-like stem cells are present in normal peripheral blood of young, middle-aged, and aged subjects. Stem Cells Int 2016; 2016: 7651645.
11. Bhartiya D. Shifting gears from embryonic to very small embryonic-like stem cells for regenerative medicine. Indian J Med Res 2017; 146: 15-21.
12. Ginani F, Soares DM, de Oliveira Rocha HA, de Souza LB, Barboza CAG. Low-level laser irradiation induces in vitro proliferation of stem cells from human exfoliated deciduous teeth. Lasers Med Sci 2018; 33: 95-102.
13. Wang Y, Huang YY, Wang Y, Lyu P, Hamblin MR. Photobiomodulation (blue and green light) encourages osteoblastic-differentiation of human adipose-derived stem cells: role of intracellular calcium and light-gated ion channels. Sci Rep 2016; 6: 33719.
14. Josselyn SA. The past, present and future of light-gated ion channels and optogenetics. eLife 2018; 7: e42367.
15. Emelyanov AN, Kiryanova VV. Photomodulation of proliferation and differentiation of stem cells by the visible and infrared light. Photomed Laser Surg 2015; 33: 164-174.
16. Pinelli R, Bertelli M, Scaffidi E. The first clinical case of dry age-related macular degeneration treated with photobiomodulation and nutraceuticals: a protocol proposal. CellR4 2020; 8: e2833.
17. Lipovski A, Oron U, Fedanken A, Lubar R. Low-level visible light (LLVL) irradiation promotes proliferation of mesenchymal stem cells. Lasers Med Sci 2013; 28: 1113-1117.
18. Zare F, Bayat M, Aliaghaei A, Piryaei A. Photobiomodulation therapy compensate the impairments of diabetic bone marrow mesenchymal stem cells. Laser Med Sci 2020; 35: 547-556.
19. Fekrazad R, Asefi S, Allahdadi M, Kalhori, KA. Effect of Photobiomodulation on Mesenchymal Stem Cells. Photomed Laser Surg 2016; 34: 533-542.
20. Park IS, Chung PS, Ahn JC, Leproux A. Human adipose-derived stem cell spheroid treated with photobiomodulation irradiation accelerates tissue regeneration in mouse model of skin flap ischemia. Lasers Med Sci 2017; 32: 1737-1746.
21. Khan I, Arany PR. Photobiomodulation Therapy Promotes Expansion of Epithelial Colony Forming Units. Photomed Laser Surg 2016; 34: 550-555.
22. Guerin CL, Blandinières A, Planquette B, Silvestre JS, Isreal-Biet D, Sanchez O, Smadja DM. Very Small Embryonic-like Stem Cells Are Mobilized in Human Peripheral Blood during Hypoxemic COPD Exacerbations and Pulmonary Hypertension. Stem Cell Rev Rep 2017; 13: 561-566.
23. Zuba-Surma EK, Kucia M, Dawn B, Guo Y, Ratajczak MZ, Bolli R. Bone marrow-derived pluripotent very small embryonic-like stem cells (VSELs) are mobilized after acute myocardial infarction. J Mol Cell Cardiol 2008; 44: 865-873.
24. Virant-Klun I, Skerl P, Novakovic S, Vrtacnik-Bokal E, Smrkolj, S. Similar Population of CD133+ and DDX4+ VSEL-Like Stem Cells Sorted from Human Embryonic Stem Cell, Ovarian, and Ovarian Cancer Ascites Cell Cultures: The Real Embryonic Stem Cells? Cells 2019; 8: 706.
25. Burd A, Zhu N, Poon VK. A study of Q-switched Nd:YAG laser irradiation and paracrine function in human skin cells. Photodermatol Photoimmunol Photomed 2005; 21: 131-137.
26. Marais A, Adams B, Ringsmuth AK, Ferretti M, Gruber JM, Hendrikx R, Schuld M, Smith SL, Sinayskiy I, Krüger T, Petruccione F, van Grondelle R. The future of quantum biology. J Royal Soc Inter 2018; 15: 20180640.