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(54) Title: INCREASE OF THE PRODUCTIVITY OF PRODUCTION CELLS BY MEANS OF LIGHT CONTROL

(57) Abstract: The present invention relates to a method for increasing the productivity of eukaryotic production cells, said production cells expressing one or more protein(s) of interest (POI) during a production phase, comprising the step of irradiating said cells with light having a wavelength or a range of wavelengths within the range of 450 to 500 nm during said production phase.

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Increase of the productivity of production cells by means of light control

The present invention relates to a method for increasing the productivity of eukaryotic production cells, said production cells expressing one or more protein(s) of interest (POI) during a production phase, comprising the step of irradiating said cells with blue light having a wavelength or a range of wavelengths within the range of 450 to 500 nm during said production phase.

Biopharmaceuticals play an important role in today's medical treatments and healthcare. The market share of biopharmaceuticals, mainly antibodies, increased vastly over the last decade, and is also forecasted to increase further in the future. Coinciding with the rising market volumes, to meet the demand productivities should equally raise. In the past, this was achieved predominately by improving volumetric productivities, *i.e.*, improvements of cultivation conditions for increasing viable cell density and volumetric product formation rates. However, such approaches soon reach technical limits of bioreactors which is why the improvement of cell-specific productivity came to the fore for researchers. Currently applied methods to achieve this are the use of chemicals, for example sodium butyrate, valproic acid, or lithium chloride; the cultivation at lower pH values; temperature shifts; or the cultivation at higher osmolality.

However, respective methods are either invasive, e.g. by way of addition of medium additives, and thus affect the processing of the product from the culture supernatant, and/or are technically complex, e.g. necessitating the cooling of the bioreactor when adjusting the temperature changes.

Thus, there still is a need for further means to increase the productivity of production cells in the production of biopharmaceuticals, in particular means that are non-invasive, do not require the addition of any substances to the cell culture, and/or do not require any genetic modifications of the cells.

Accordingly, the technical problem underlying the present invention is the provision of methods for increasing the productivity of production cells in the production of biopharmaceuticals, preferably fulfilling the above requirements.

- 5 The solution to the above technical problem is achieved by the embodiments characterized in the claims.

In particular, in a first aspect, the present invention relates to a method for increasing the productivity of production cells, said production cells expressing one or more
10 protein(s) of interest (POI) during a production phase, comprising the step of irradiating said cells with light having a wavelength or a range of wavelengths within the range of 450 to 500 nm during said production phase.

The term “productivity” as used herein refers to the cell-specific productivity of the
15 production cells as far as production, *i.e.*, expression, of the one or more POIs is concerned. Thus, cell-specific production is defined as the amount of expressed POI(s) (e.g. in pg of expressed protein(s)) per cell per day.

The eukaryotic production cells used in the present invention are not particularly
20 limited and include any type of eukaryotic cells capable of expressing one or more given POIs. Suitable eukaryotic cells are again not particularly limited and include e.g. yeast cells, insect cells, avian cells, and mammalian cells, wherein mammalian cells are preferred. Suitable mammalian cells are also not particularly limited and include any mammalian cell lines suitable for the expression of recombinant proteins.
25 Respective examples include CHO cells (e.g. CHO DP12 cells), HEK293 cells, COS cells, Vero cells, HeLa cells, and NS0 cells, wherein CHO cells, in particular CHO DP12 cells, are preferred.

POIs that can be expressed in the methods of the present invention are not
30 particularly limited and include any POIs expression of which is desired and possible by way of expression in cells. Of particular interest in this respect are antibodies and antibody derivatives which are preferred.

According to the present invention, the production cells are irradiated with light having a wavelength or a range of wavelengths within the range of 450 to 500 nm during said production phase. In preferred embodiments, the light with which the production cells are irradiated has an average intensity of 9 to 15 W/m², preferably 10 to 14 W/m², more preferably 11 to 13 W/m², more preferably about 12 W/m². In this context, the term "average intensity" reflects the fact that the light installation for cell cultures might not allow for equal light distance for all sides of the culture vessel. In further preferred embodiments, the irradiation with light exposes the production cells to a light dose of 3 to 5 W×h/m², preferably about 4 W×h/m². Irradiation with light can be intermittent or over extended periods of time, provided that the above average intensity and light dose are observed.

According to the present invention, light having a wavelength or a range of wavelengths within the range of 450 to 500 nm, preferably 450 to 480 nm, more preferably 450 to 460 nm, is used for irradiating the production cells. In preferred embodiments, light having a wavelength of about 455 nm, e.g. of 455 nm, is used.

The source of light for generating the irradiating light is not particularly limited and includes any light sources capable of producing light with the desired wavelength or in the desired wavelength range. Preferably, the light is generated by light-emitting diodes (LEDs).

In specific embodiments, the production cells are not genetically modified to express a light sensitive protein. In particular, the productivity increasing effect of the methods of the present invention is expressly not dependent on such light sensitive proteins. However, in other embodiments, production cells that are genetically modified to express a light sensitive protein can be used, as the productivity increasing effect of the methods of the present invention can be more pronounced in this case. Means for generating genetically modified cells expressing a light sensitive protein, as well as suitable light sensitive proteins, are not particularly limited and are known in the art.

Types of cell culture amendable to the present invention are not particularly limited. However, in the context of biopharmaceuticals production, usually cells are cultivated in suspension culture or adherent culture, wherein suspension culture is preferred. Further, cells can be kept in a batch cell culture, a fed-batch cell culture
5 or a perfusion cell culture, and are usually kept in a perfusion cell culture, e.g. in a perfusion bioreactor. Respective cell culture techniques and bioreactors are known in the art.

Means of irradiating production cells in a cell culture during the production phase
10 are also not particularly limited and are known in the art. However, in preferred embodiments, the production cells are kept in a bioreactor and the irradiation of the production cells with light takes place at a circulation tube of the bioreactor. Such circulation tubes are typically only a few cm in diameter, even in large scale production bioreactors, which facilitates a uniform light irradiation.

15 As used herein, the term “comprising”/“comprises” expressly includes the terms “consisting essentially of”/“consists essentially of” and “consisting of”/“consists of”, *i.e.*, all of said terms are interchangeable with each other herein.

20 Further, as used herein, the term “about” represents a modifier of $\pm 10\%$ of the specified value, preferably $\pm 7.5\%$, $\pm 5\%$, $\pm 3\%$, $\pm 2\%$, or $\pm 1\%$ of the specified value. Thus, by way of example, the term “about 10” includes the ranges 9 to 11, 9.25 to 10.75, 9.5 to 10.5, 9.7 to 10.3, 9.8 to 10.2, and 9.9 to 10.1.

25 The present invention found that the productivity, *i.e.*, cell-specific productivity of production cells expressing one or more protein(s) of interest, e.g. antibodies or other biopharmaceuticals, can be advantageously increased by way of irradiating the production cells with blue light, in accordance with the present invention, during the protein production phase.

30 In contrast to other known methods, the methods of the present invention represent a non-invasive control measure which, moreover, does not require genetic modification of the production cells. Through targeted light irradiation, cells are

arrested in the cell cycle, so that the non-dividing cells put their production output into the over-production of proteins. This positive effect was not expected in this way. It is known from the prior art that irradiation with certain wavelengths, especially with higher-energy blue light, has rather harmful effects on cells. In fact, there are various publications in which irradiation with blue light is used to kill cancer cells, for example. Furthermore, it was expected that the cells would need certain optogenetic "light sensors" in order to be susceptible to light induction. Surprisingly, the present inventors found that the cells are susceptible to blue light even without optogenetic modifications and that the targeted blue irradiation is conducive to the overexpression of proteins.

Investigations of blue light have been conducted usually in relation to plants or concerning its effect on skin or retinal cells. For cells of a producing cell line, the effect of blue light, so far, has only been shown to have a negative impact on viability and cell growth. This effect has been shown at relatively low intensities of 0.5 – 2.5 W/m² and after 24 hours, being exposed to a light dose of 12 to 60 W/m² per day.

In other instances, where a connection between blue light and productivity was found, it is only in conjunction with optogenetic switches. For this, organisms are genetically altered to express a light sensitive protein with a protein of interest. These proteins undergo a conformational change when illuminated with the corresponding wavelength. This can be coupled in different ways, for example to induce a production pathway, to control the flux in a metabolism pathway, or induction of cell lysis to facilitate downstream processing.

Surprisingly, in the present invention, it could be shown that cells are not dying after being illuminated with blue light for a longer period of time with an intensity of 12 W/m², resulting in a light dose of 96 W/m² per day. On the contrary, it could be shown that the illumination of the cells resulted in an improved cell-specific productivity compared to control cells which were cultivated without the addition of blue light.

The figures show:

Figure 1:

Cell-specific productivity of CHO DP12 cells illuminated by blue LED light. DC = dark control, BL = blue light. Error bars are SD of n=3 biological replicates.

Figure 2:

Percentage of CHO DP12 cells in the G1-phase of the cell cycle after the indicated time of illumination with blue LED light compared to control cells kept in the dark. Error bars are SD of n=3 biological replicates.

Figure 3:

SEAP activity (U/l) per cell of CHO cells transfected with a plasmid containing SEAP after illumination in a 96-well plate for 48 h with a blue LED. DC = dark control, blue bars indicate the illumination with blue light. Error bars are SD of n=4. P values were calculated using a Students t-test (**P < 0.01).

Figure 4:

Cell-specific productivity of CHO DP12 cells illuminated by red LED light. Cells were kept in the dark (DC) or illuminated with red light (RL). Error bars are SD of n=3 biological replicates.

Figure 5:

Cell-specific productivity of CHO DP12 cells illuminated by blue LED light in different intensities and illumination durations. Cells were kept in the dark (Dark Control) or illuminated with blue light with an intensity of 3, 6 or 12 W/m² and with a duration of 0.33, 0.166 or 0.0166 hours per hour. Error bars are SD of n=3 biological replicates.

Figure 6:

Cell-specific productivity of CHO DP12 cells in batch mode. Cells were kept in the dark (DC) or illuminated with blue LED light (BL) installed at the connection tube between the bioreactor and the hollow fiber module. Error bars are SD of n=2 biological replicates.

Figure 7:

Cell-specific productivity of CHO DP12 cells in fed-batch mode. Cells were kept in the dark (DC) or illuminated with blue LED light (BL) installed at the connection tube between the bioreactor and the hollow fiber module. Error bars are SD of n=2 biological replicates.

Figure 8:

Cell-specific productivity of CHO DP12 cells in perfusion mode. Cells were kept in the dark (DC) or illuminated with blue LED light (BL) installed at the connection tube between the bioreactor and the hollow fiber module.

The present invention will be further illustrated by the following examples without being limited thereto.

Examples

Material and methods:*Mini bioreactor cultivation of cells*

IgG1 producing CHO DP-12 cells were cultured in a shaking incubator at 150 rpm with 50 mm displacement (minitron, infors AG) at 37°C, 5% CO₂ in a humid atmosphere. Chemically defined TC-42 medium (Xell AG) in 50 mL mini bioreactors (Corning GmbH) with a filter cap were used. Cells were seeded at 0.2×10^6 cells/mL. Viable cell density was measured each day via holographic microscopy (fluidlab-300, anvajo GmbH).

Illumination of cells in the shaking incubator

Waterproof LED strips (VARDAflexIP68, rutc Licht GmbH & Co. KG) were wrapped around the reaction cup holder, connected to a Raspberry Pi 4 (Raspberry Pi

Foundation) and controlled via a Python script (Python Software Foundation, <https://www.python.org/>). Intensities of 3 W/m², 6 W/m² or 12 W/m² and intervals of 1 min illumination, 29 min dark phase (1/29); 5 min illumination, 25 min dark phase (5/25); and 10 min illumination, 20 min dark phase (10/20) were examined.

- 5 Intensities are averages, as the light installation for suspension cultures does not allow for equal light distance for all sides of the mini bioreactors.

Batch cultivation with an attached ATF2 module

- 10 For batch cultivations, a 4 L DasGIP bioreactor (Eppendorf SE) with a working volume of 3 L was used. Cultivations were realized with a controlled pH of 7.1, temperature of 37°C and dO of 40%. Agitation speed was set at 200 rpm and a combination of rushton and impeller stirrer blades was used. A hollow fiber module (Cytiva Europe GmbH) as a part of an alternating tangential flow set up (Xcell ATF2, Repligen Corporation) was attached to the bioreactor. The regular silicone tube connection was replaced with a glass tube with the same dimensions for a uniform illumination of the cells. The LED lights were installed around the glass tube and the ATF module was turned on 24 hours after inoculation.

20 *Fed-batch cultivation with an attached ATF2 module*

- For fed-batch cultivations, a 4 L DasGIP bioreactor (Eppendorf SE) with a working volume of 3 L was used. Cultivations were realized with a controlled pH of 7.1, temperature of 37°C and dO of 40%. Agitation speed was set at 200 rpm and a combination of rushton and impeller stirrer blades was used. Feed started 72 hours after inoculation. A hollow fiber module (Cytiva Europe GmbH) as a part of an alternating tangential flow set up (Xcell ATF2, Repligen Corporation) was attached to the bioreactor. The regular silicone tube connection was replaced with a glass tube with the same dimensions for a uniform illumination of the cells. The LED lights were installed around the glass tube and the ATF module was turned on 72 hours after inoculation.

Alternating-tangential flow perfusion cultivation with an ATF2 module

For perfusion cultivations, a 4 L DasGIP bioreactor (Eppendorf SE) with a working volume of 3 L was used. Cultivations were realized with a controlled pH of 7.1, temperature of 37°C and dO of 40%. Agitation speed was set at 200 rpm and a combination of rushton and impeller stirrer blades was used. A hollow fiber module (Cytiva Europe GmbH) as a part of an alternating tangential flow set up (Xcell ATF2, Repligen Corporation) was attached to the bioreactor. The regular silicone tube connection was replaced with a glass tube with the same dimensions for a uniform illumination of the cells. The LED lights were installed around the glass tube. Perfusion was started 72 hours after inoculation with 0.7 VVD.

Cell cycle analysis

1×10^6 cells were washed in ice-cold PBS, fixed with ice-cold fixation buffer (70% ethanol, 30% PBS) and stored at -20°C until investigation. Cells were washed twice with PBS + 1% albumin and the cell pellet was resuspended in staining buffer (propidium iodide (PI) and RNase in PBS). After 10 min incubation in the dark, the cells were examined with a MACSQuant Analyzer with a 610/20 nm filter and 50000 events. Data were analyzed with FACSalyzer software.

Enzyme-linked immunosorbent assay against IgG-antibody

The concentration of secreted antibody was determined with an enzyme-linked immunosorbent assay (ELISA). High-binding 96-well plates were coated with an anti-human IgG F(c) (goat) antibody (Rockland Immunochemicals). Free binding sites were blocked with 1% BSA in TBS. Diluted supernatants and standards were pipetted into the coated wells in duplicates. Detection was done with a horseradish peroxidase coupled anti-human kappa chain (goat) antibody (Rockland Immunochemicals) and TMB substrate (SeramunBlau, Seramun Diagnostica). After stopping the reaction with 0.25 M sulphuric acid, the absorption was measured at 450 nm (Tecan Spark, Tecan Trading, Ltd.). Background was measured at 620 nm and subtracted from the values at 450 nm.

Example 1:Positive effect of blue LED light on cell-specific productivity of antibody producing CHO DP12 cells

5 IgG1 producing CHO DP12 cells were cultivated as suspension culture in a chemically defined, animal-component free medium (TC-42, Xell AG) and illuminated with blue LED light. Cultivation was realized in 50 mL mini bioreactors with a working volume of 15 mL in a shaking incubator with a 5% CO₂ overlay at 37°C in a humid atmosphere. The LEDs, in form of a commercially available LED
10 strip, were wrapped around the test tube holder. The light installation was realized in a way that a preferably uniform illumination is achieved. Cells were illuminated with a total light dose of 4 W×h/m².

Antibody titers were quantified via enzyme-linked immunosorbent assay (ELISA).
15 The cell-specific productivity (csp) was calculated as pg per cell per day. Cell cycle analysis was conducted via flow cytometry and propidium iodide staining.

Illumination of cells with blue LED light (BL) raised the csp around 60% compared to the control cells (DC) (Fig. 1).
20

Various other studies have shown that cell-specific productivity coincides with the number of cells in the G1-phase of the cell cycle.

Cell cycle observations conducted in these experiments were congruent with the
25 aforementioned csp improvement. Cells exposed to blue light had a significant higher number of cells in the G1-phase of the cell cycle after 72 h (Fig. 2).

Example 2:

The positive effect of blue light on the productivity of SEAP in CHO cells is dependent on the used intensity

- 5 Adherent CHO K1 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin (100 U/ml)/streptomycin (100 µg/ml) in a humidified 5% CO₂ atmosphere at 37°C in 100 mm TC-treated culture dish. 24 h after seeding in a black 96-well plate the cells were transfected with Lipofectamine 3000™ following the manufacturer's protocol
- 10 with a plasmid containing constitutively expressing secreted alkaline phosphatase (SEAP). For the next 24 h the cells were kept in dark before being illuminated with the optoPlate-96. The cells were either kept in the dark or illuminated with 0.44 W×h/m² or 4 W×h/m² for the next 48 h. Afterwards the supernatant of the cells was used to determine the SEAP activity following protocol known in the art and the
- 15 cell count was determined using a CASY counter.

When the cells were illuminated with 4 W×h/m² of blue light a significant increase of SEAP activity per cell compared to the dark control can be observed. However, this effect was not observable when using 0.44 W×h/m² of blue light, indicating that the

20 intensity of blue light is relevant for inducing the effect on the productivity of the cells (Fig. 3)

Example 3:

- 25 Red LED light has no effect on cell-specific productivity

IgG1 producing CHO DP12 cells were cultivated as suspension culture in a chemically defined, animal-component free medium (TC-42, Xell AG) and illuminated with red LED light. Cultivation was realized in 50 mL mini bioreactors

30 with a working volume of 15 mL in a shaking incubator with a 5% CO₂ overlay at 37°C in a humid atmosphere. The LEDs, in form of a commercially available LED strip, were wrapped around the test tube holder. The light installation was realized

in a way that a preferably uniform illumination was achieved. Cells were illuminated with a total light dose of $\sim 4 \text{ W}\times\text{h}/\text{m}^2$.

Antibody titers were quantified via enzyme-linked immunosorbent assay (ELISA).

5

Illumination of cells with red LED light (RL) had no positive effect on csp compared to the control cells (DC) (Fig. 4).

10 Example 4:

Light intensity and illumination duration play a key role for the positive effect on csp of CHO DP12 cells

IgG1 producing CHO DP12 cells were cultivated as suspension culture in a chemically defined, animal-component free medium (TC-42, Xell AG) and illuminated with blue LED light. Cultivation was realized in 50 mL mini bioreactors with a working volume of 15 mL in a shaking incubator with a 5% CO₂ overlay at 37°C in a humid atmosphere. The LEDs, in form of a commercially available LED strip, were wrapped around the test tube holder. The light installation was realized in a way that a preferably uniform illumination is achieved.

20

Three different light intensities and illumination durations were tested, resulting in different total light doses (Table 1). For the LED control, a Raspberry Pi 4 and a Python script were used.

25

Antibody titers were quantified via enzyme-linked immunosorbent assay (ELISA). The cell-specific productivity (csp) was calculated as pg per cell per day.

Only the highest total light dose of $4.0 \text{ W}\times\text{h}/\text{m}^2$ induced a significant raise of the csp in CHO DP12 cells (Fig. 5).

30

Table 1: Tested illumination conditions and the resulting light doses.

Light intensity (W/m ²)	Frequency Light/Dark (min)	Illumination time (h)	Total light dose (W×h/m ²)
~12	10/20	0.33	4.0
	5/25	0.166	2.0
	1/29	0.0166	0.2
~6	10/20	0.33	2.0
	5/25	0.166	1.0
	1/29	0.0166	0.1
~3	10/20	0.33	1.0
	5/25	0.166	0.5
	1/29	0.0166	0.05

Example 5:

5 Raise of the csp of CHO DP12 cells in a perfusion reactor set up and blue LED light

IgG1 producing CHO DP12 cells were cultivated as suspension culture in batch mode, fed-batch mode or perfusion mode in a chemically defined, animal-component free medium (TC-32A, Xell AG) and illuminated with blue LED light.

10 Cultivation was realized in a 4 L DasGIP bioreactor with a working volume of 3 L at 37°C and a controlled pH set point at 7.1. An ATF2 module was connected to the reactor as means to install the LED light in a defined manner. The LEDs, in form of a commercially available LED strip, were wrapped around the connection tube between the reactor and a hollow fiber module.

15

First experiments, without any optimization, show that blue light installed at a stirred tank reactor raises the csp around 15% when cells are cultivated in batch mode (Fig. 6), around 35% in fed-batch mode (Fig. 7), and around 45% in perfusion mode (Fig. 8).

20

Claims

1. A method for increasing the productivity of eukaryotic production cells, said production cells expressing one or more protein(s) of interest (POI) during a production phase, comprising the step of irradiating said cells with blue light having a wavelength or a range of wavelengths within the range of 450 to 500 nm during said production phase.
2. The method of claim 1, wherein the light with which the production cells are irradiated has an average intensity of 9 to 15 W/m².
3. The method of claim 1 or claim 2, wherein the irradiation with light exposes the production cells to a light dose of 3 to 5 W×h/m².
4. The method of any one of claims 1 to 3, wherein the light has a wavelength of about 455 nm.
5. The method of any one of claims 1 to 4, wherein the eukaryotic production cells are mammalian cells.
6. The method of claim 5, wherein the mammalian cells are CHO cells.
7. The method of claim 6, wherein the CHO cells are CHO DP12 cells.
8. The method of any one of claims 1 to 7, wherein the production cells are not genetically modified to express a light sensitive protein.
9. The method of any one of claims 1 to 8, wherein the productivity is the cell-specific productivity expressed as amount of expressed POI per cell per day.
10. The method of any one of claims 1 to 9, wherein the production cells are cultivated in suspension culture.

11. The method of any one of claims 1 to 9, wherein the production cells are cultivated in adherent culture.
12. The method of any one of claims 1 to 11, wherein the production cells are cultivated in a batch cell culture, a fed-batch cell culture, or a perfusion cell culture.
13. The method of claim 12, wherein the production cells are cultivated in a perfusion cell culture.
14. The method of claim 13, wherein the production cells are cultivated in a perfusion bioreactor.
15. The method of any one of claims 1 to 14, wherein the production cells are cultivated in a bioreactor and wherein irradiation of the productions cells with light takes place at a circulation tube of said bioreactor.

Figures

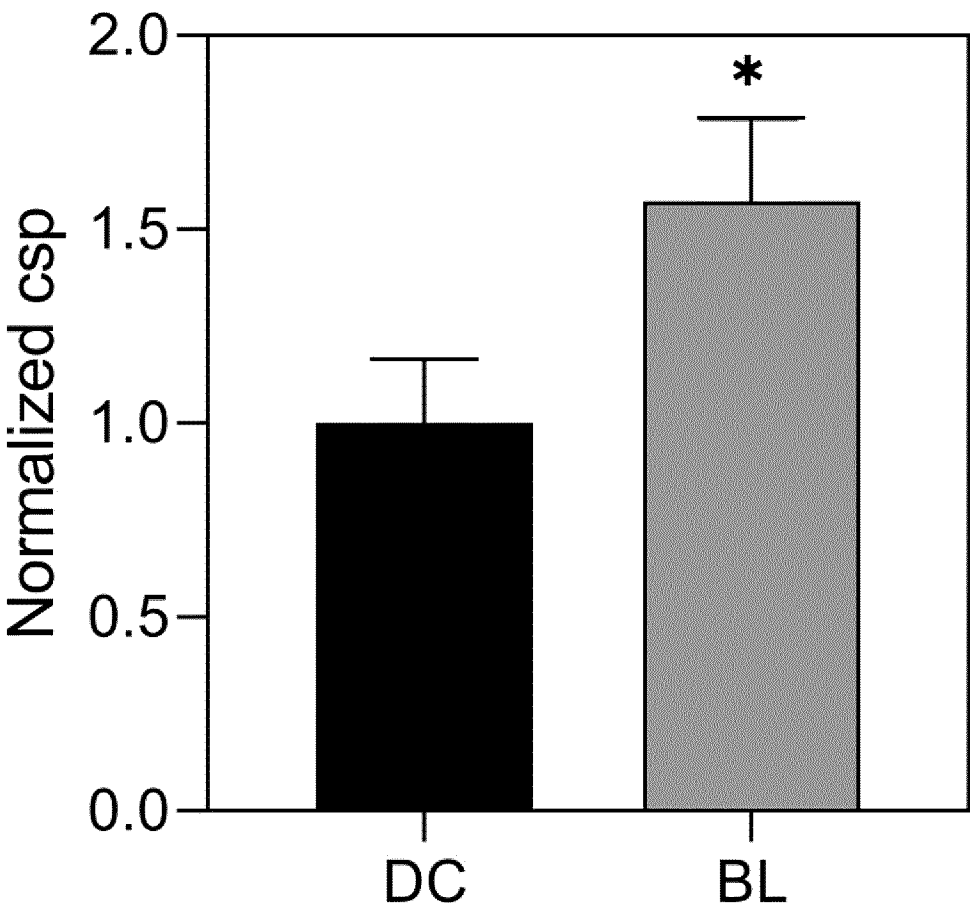


Figure 1

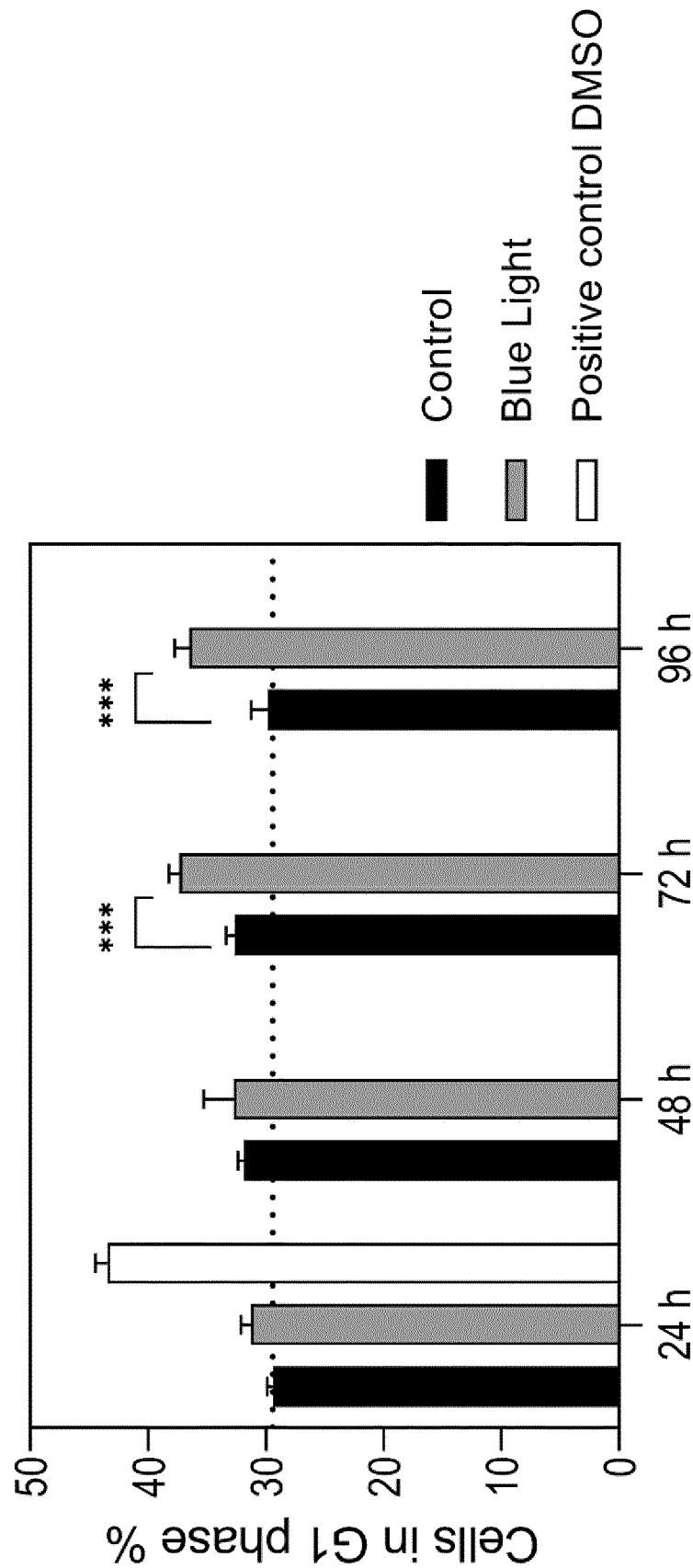


Figure 2

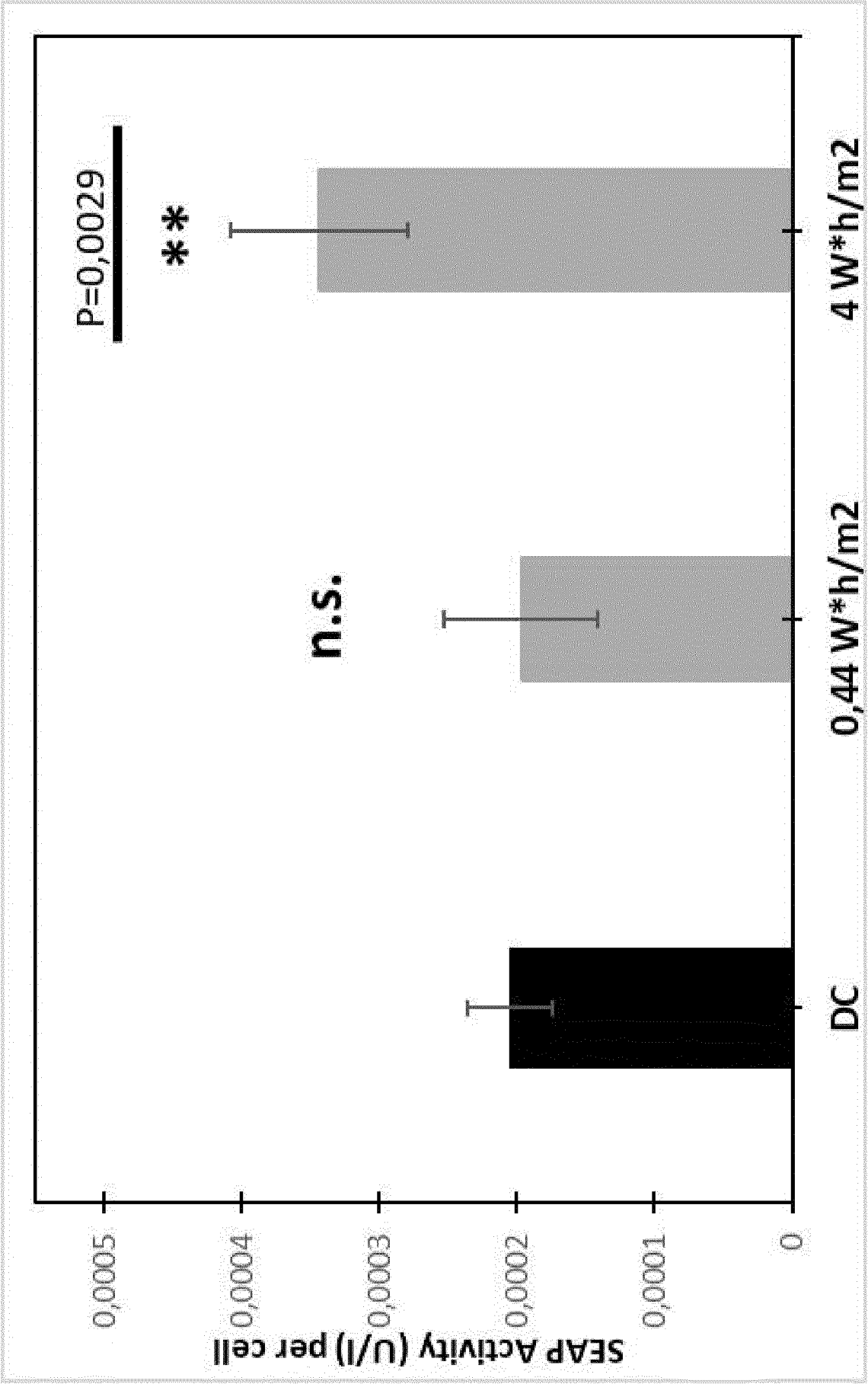


Figure 3

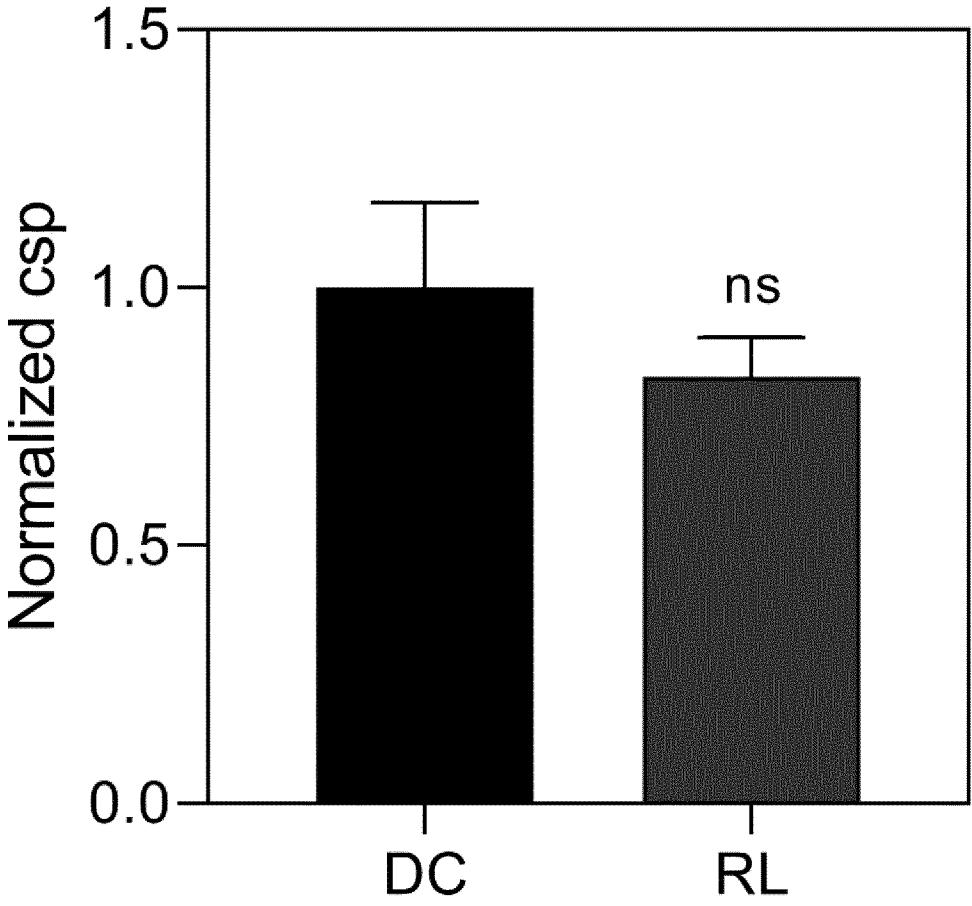


Figure 4

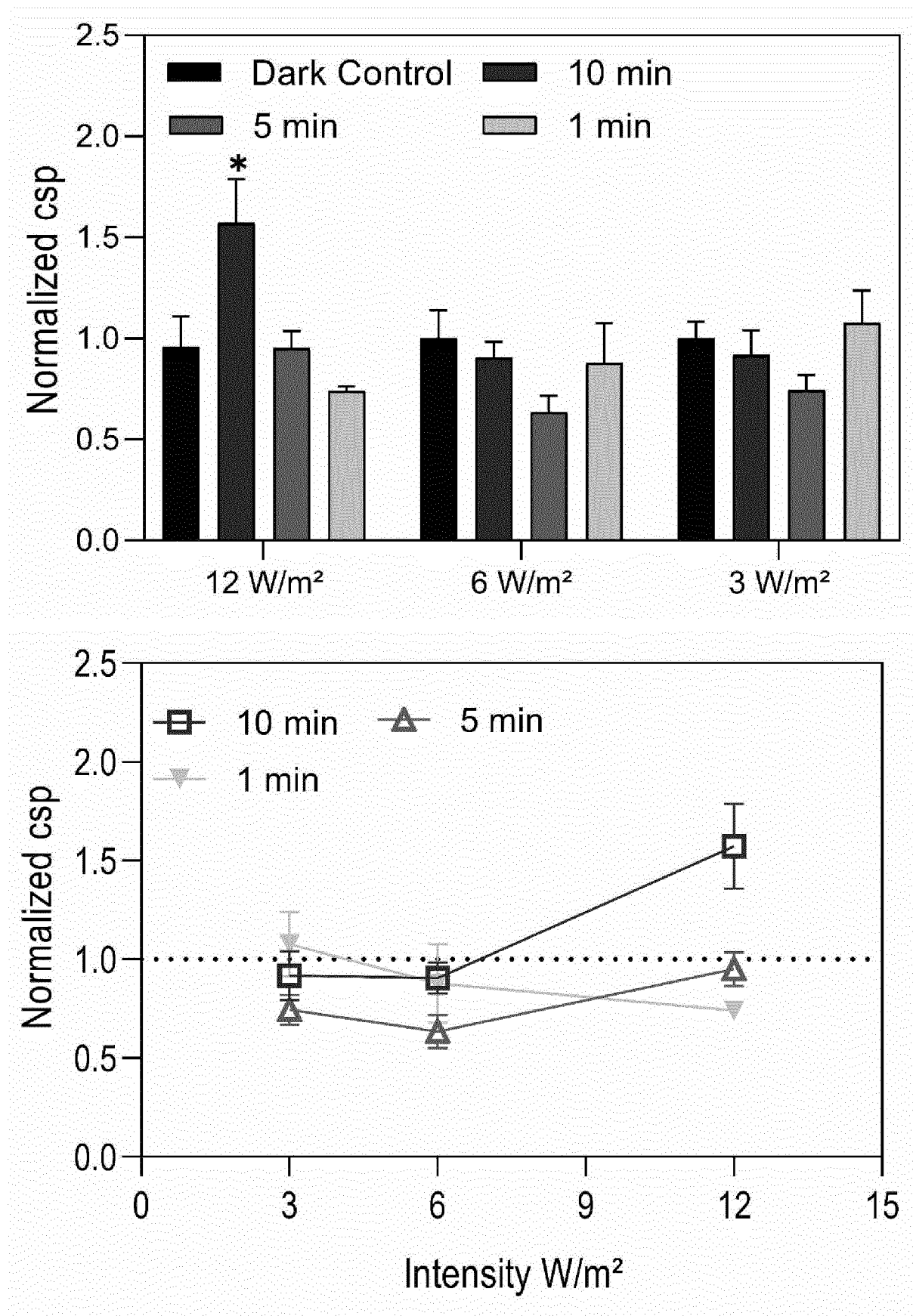


Figure 5

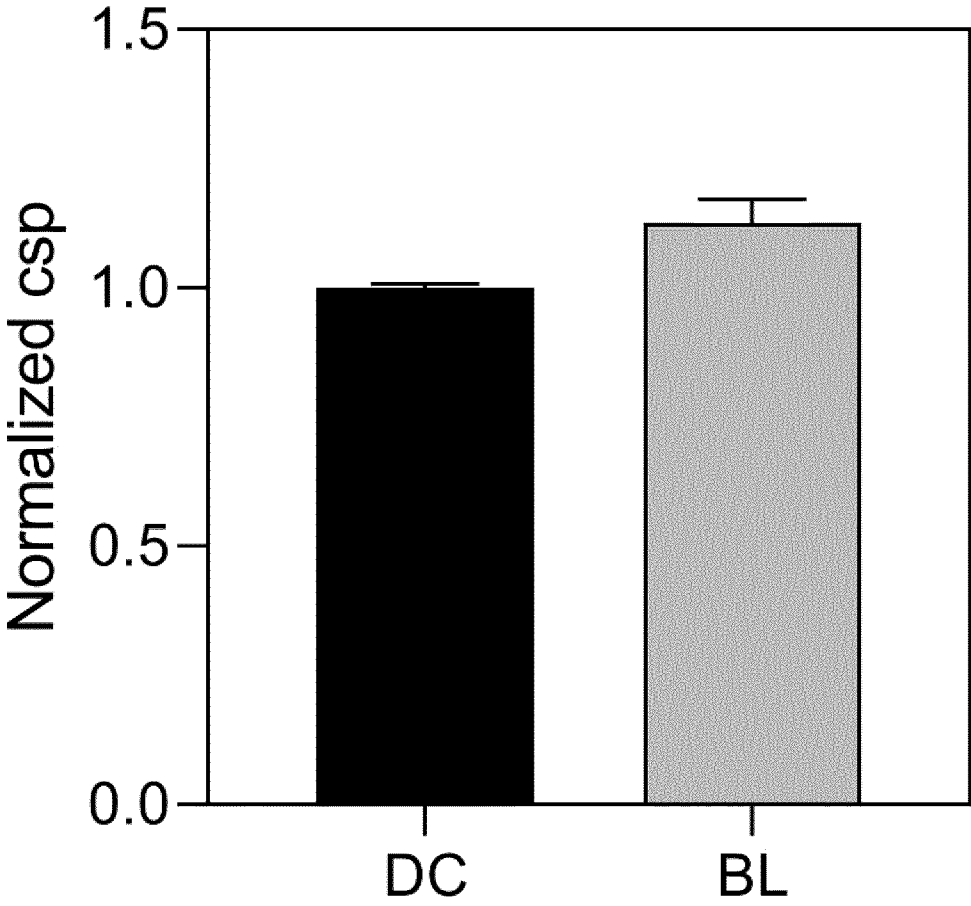


Figure 6

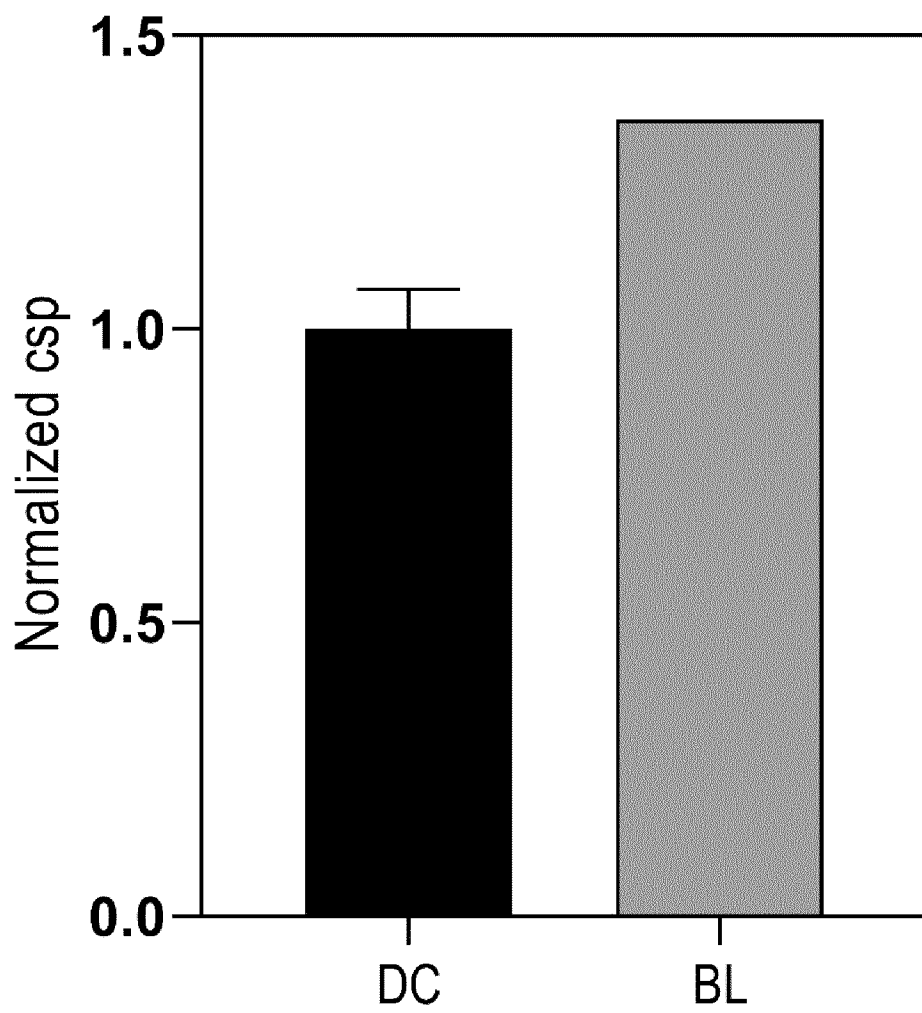


Figure 7

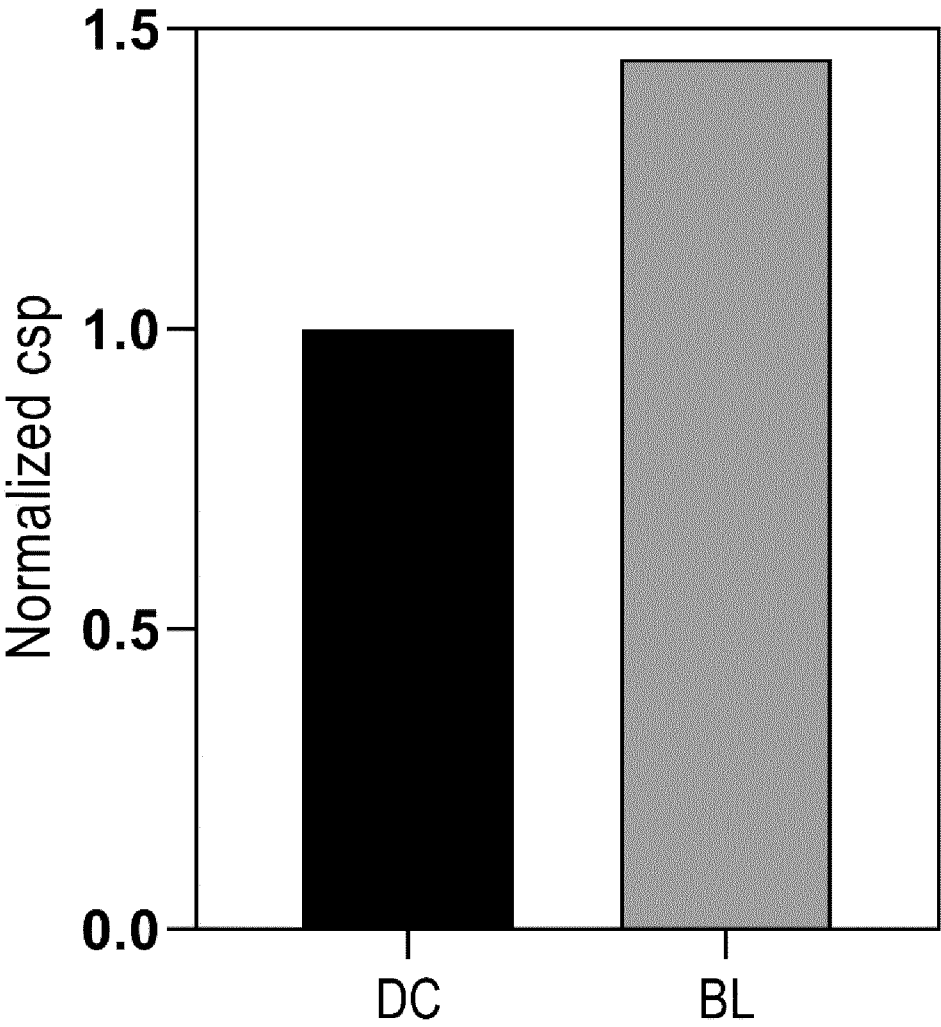


Figure 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/058109**A. CLASSIFICATION OF SUBJECT MATTER**INV. C12N5/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHEDMinimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/223155 A1 (STREETER JACKSON [US]) 5 October 2006 (2006-10-05) claims 4, 21 -----	1 - 3
A	YOO JU AH. ET AL: "Blue Light Irradiation Induces Human Keratinocyte Cell Damage via Transient Receptor Potential Vanilloid 1 (TRPV1) Regulation", OXIDATIVE MEDICINE AND CELLULAR LONGEVITY, vol. 2020, 16 December 2020 (2020-12-16), pages 1-14, XP093178091, US ISSN: 1942-0900, DOI: 10.1155/2020/8871745 Retrieved from the Internet: URL:http://downloads.hindawi.com/journals/ omcl/2020/8871745.xml> material and methods; figures 1, 2 ----- - / - -	1 - 15



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/058109

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Information on patent family members

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