

Three-Dimensional Scaffold, Light Emitting Diode (LED) And Melatonin Effects on Cell Proliferation and Testosterone Secretion in TM3 Leydig Cells

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Abstract

Different effects of fibrin scaffold, green LED and melatonin on cell proliferation and differentiation have received extensive attentions. Testosterone, the most important sex hormone is secreted by Leydig cells to regulate spermatogenesis and many other phenomena in the body organs. The aim of the present study was to evaluate the proliferation of Leydig cells and their testosterone secretion under the influence of three-dimensional scaffold culture, green light irradiation and melatonin. The experimental groups, were TM3 cells incorporated in 3D scaffold culture, exposed to green light and also exposed to melatonin individually, in pairs and three factors together. Cell proliferation and testosterone secretion were measured after 72 hr. Cell cycle genes including PCNA, CYCLIND1, CDC2 and CDKN1B and, testosterone related genes; GATA4 and ROR α were also examined. In general, three-dimensional scaffold increased Leydig cells proliferation, testosterone secretion and the expression of the related genes. On the other hand, melatonin decreased cell proliferation and testosterone synthesis. Green light did not significantly change the results but slightly decreased cell proliferation and testosterone synthesis. Combination of green light with melatonin significantly reduced the outcome while, light with scaffold increased the results. Application of light + scaffold + melatonin covered the decreasing effect of melatonin.

Introduction

Testes, the organs of male sex hormones secretion, play an important role in the maintenance and maturation of germ cells. Leydig and Sertoli cells of testes, work together to orchestrate and regulate spermatogenesis¹. Leydig cells promote the proliferation and development of germ cells in the seminiferous tubules and maintain the high level of androgen concentrations including testosterone^{2,3}; the most abundant sex hormone in the body, which regulates spermatogenesis. In the absence of testosterone or androgen receptors, spermatogenesis will cease at the meiotic stage⁴.

Melatonin, a lipophilic and hydrophilic hormone which is mainly secreted by the pineal gland is involved in the regulation of circadian rhythms. It could pass through the blood–testis barrier, enter the testicular cells and act through specific receptors including membrane melatonin receptors 1 (MT1) and 2 (MT2). MT1 and MT2 play some role in testosterone synthesis by regulating cAMP signal transduction cascades thorough coupling with G-protein. Melatonin can bind to retinoic acid receptor-related orphan receptor A (ROR α), which has been identified in many mammalian cell types. ROR α regulates the levels of reproductive hormones and animal reproduction at the transcriptional level through a nuclear receptor depending on physiological conditions and species of animals⁵.

In vitro three-dimensional (3D) culture mimics the physiologic milieu of the *in vivo* conditions as it could fill the gap between two-dimensional (2D) cell culture and animal tissues, and has been suggested as a solution for 2D culture insufficiencies⁶. To achieve a 3D system, the cells are seeded onto a porous matrix, known as scaffolds, to attach and colonize⁷. The most common materials utilized as scaffold are metals such as titanium; synthetic organic materials such as polymers; synthetic inorganic materials such as hydroxyapatite, and natural organic materials such as collagen and fibrin⁸. Fibrinogen is a blood

plasma glycoprotein that is essential for wound healing and other biological phenomena. Fibrinogen-based 3D scaffold is a biologic network assembled by fibrinogen polymerization⁹ in which stem and somatic cells proliferate and differentiate more successfully¹⁰.

Some physical factors including light emitting diodes (LEDs) irradiations have currently been introduced to potentially affect proliferation and differentiation of various cell types, mostly the mesenchymal stem cells^{11,12}. Although the mechanism is not fully revealed, it has been postulated to be due to an increase in the oxidative function of mitochondria¹³⁻¹⁷. Considering the significance of testosterone in sexual activities and the probable effect of melatonin on the function and level of testosterone, and also the effects of 3D culture and green light emitted from a LED, on the proliferation and differentiation of different cells, the aim of the present study was to examine the effects of melatonin, 3D culture condition and green light irradiation on the cell proliferation, testosterone secretion and gene expression of a cellular model of testosterone production; TM3 cells.

Results

Cell proliferation assay by MTT

Proliferation of TM3 cells in the different groups was evaluated using MTT assay. Each of the interventions including melatonin administration and LED irradiation reduced the cell proliferation rate compared with the control group. The least proliferation rate was detected in LED+MEL group (47%, $P<0.001$), followed by LED (75%, $P<0.001$) and MEL (97%) groups. The use of scaffold significantly increased cell proliferation rate in the related groups. The highest proliferation rate was observed in SC+MEL group (147%, $P<0.001$), followed by SC and LED+MEL+SC (137%, $P<0.001$), and LED+SC (125%, $P<0.01$) (Figure 1).

Trypan blue cell viability assessment

Cell viability was measured using trypan blue staining method in which the highest cell viability rate (93%) was in CTRL group, while the lowest viability rate was detected in MEL group (76%, $P<0.001$), followed by LED+MEL (77%, $P<0.001$) and LED (81%, $P<0.01$) None of the treatments resulted in a significant difference among the treated groups (Figure 2). It should be noted that the viability assay was not performed in the scaffold-containing groups due to the limitations of trypan blue method.

Testosterone level in the different groups

The amount of testosterone in the supernatant of the cultured TM3 cells in all groups was measured 24 hours after a hCG challenge, using an ELISA kit. The highest amount of testosterone was detected in LED + SC group (3634 ± 285 ng/l), followed by 3614 ± 220 ng/l for LED + MEL + SC, 3563 ± 91 ng/l for LED + MEL, 3389 ± 367 ng/l for CTRL, 3338 ± 132 ng/l for LED, 3328 ± 204 ng/l for SC and 3134 ± 71 ng/l for SC +MEL groups. However, the values in the various groups were not significantly different compared with the control and other treatment group (Figure 3).

Gene expression analysis

Expression of different genes associated with the cell cycle and testosterone synthesis was evaluated by qRT-PCR. The expression pattern of the genes that are involved in the cell cycle mechanism including PCNA, CYCLIN D1 and CDC2 was evaluated and the results showed that exposure of TM3 cells to a 3D condition generally increased the expression level of these genes compared with the scaffold-free environments (2D) (Figure 4: a, b and c).

The expression level of PCNA gene, which is involved in the stage S of the cell cycle, increased in the scaffold-containing groups compared with the control group and the scaffold-free groups. It was significantly ($P < 0.05$) higher in the SC + Mel group compared with the MEL group and also it was significantly ($P < 0.05$) higher in the LED+ SC+ MEL group compared with the control group ($P < 0.05$) (Figure 4, a).

The expression level of CDC2 gene which encodes cdk1 protein and is highly involved in cell cycle progression, increased in the scaffold-containing groups compared with the scaffold-free groups. It was significantly ($P < 0.05$) different in the SC group compared with the control group. It also decreased significantly and nonsignificantly in the LED + MEL ($P < 0.05$), LED ($P < 0.05$) and MEL groups compared with the control group (Figure 4, b).

The expression level of CYCLIN D1 gene, which is involved in the promotion of G1 to S stage transition, non-significantly increased in the scaffold-containing groups compared with the scaffold-free and the control groups (Figure 4, c).

The expression level of CDKN1B gene, that controls the cell cycle progression at G1 through its inhibitory effect on Cyclin D1, were contrary to the results of the Cyclin D1 gene expression as in the almost all scaffold-containing groups it significantly ($P < 0.05$) reduced compared with the scaffold-free and the control groups (Figure 4, d).

The expression of the genes involved in steroidogenesis procedures including GATA4 and ROR α increased significantly in the scaffold-containing groups, compared with the control and scaffold-free groups. ROR α expression was significantly ($P < 0.05$) higher in the LED + SC + MEL group compared with the LED + MEL and the control group. The Expression of GATA4 also followed the pattern of gene expression of ROR α . Similarly, scaffold-containing groups had higher expression of GATA4 in comparison with the scaffold-free groups. The highest expression was detected in the LED+SC+MEL group followed by SC, SC+MEL and LED+SC groups (Figure 5: a, b).

Discussion

Androgenic hormones including testosterone have a pivotal role on the development and progression of male primary and secondary sexual characteristics and also sexual activities in life. Since many factors are involved in the steroidogenesis procedures which could affect testosterone production and function,

in the present study, we investigated the proliferation and testosterone production of TM3 Leydig cells in the three-dimensional culture, green light irradiation, and melatonin administration.

Proliferation of Leydig cells in 2D and 3D culture conditions, was evaluated by MTT assay and q-RT-PCR analysis of the genes involved in the cell cycle including PCNA, CDC2, CYCLIND1, and its inhibitor: CDKN1B. Comparison of the results obtained from 2D culture with 3D culture indicated an enhancement in the proliferation of TM3 cells in the 3D culture. This increase was also observed after combination of 3D culture with LED irradiation or melatonin supplementation. In agreement with our results Bayat et al (2016), reported an increase in the stem cell proliferation and differentiation in a fibrin scaffold¹⁹. Also, Seyedi et al (2017) found that fibrin scaffold could enhance the differentiation of human umbilical cord matrix-derived stem cells into insulin-secreting cells²⁰. Based on the results of the MTT assay, fibrin scaffold could increase the proliferative capacity of TM3 cells and on the other hand, the gene expression pattern of cell cycle genes showed a parallel increase in the expression of proliferative genes with cell proliferation. But it is not clear which of these factors has the greatest influence on the increase of the testosterone secretion. However, because ROR α and GATA4 expression was higher in the scaffold-containing groups, it can be postulated that a 3D culture has enhanced cell steroidogenesis activity, regardless of cell number. Of course, measuring the total DNA content of the cells and comparing it in the different groups would provide some useful data which is absent from our work. On the other hand, the effect of 3D culture on the amount of testosterone secreted in the supernatant and especially the expression of genes involved in this process including GATA4 and ROR α , was increasing, though the serum level of testosterone secreted in the supernatant did not exactly parallel the gene expression pattern. Since testosterone synthesis from TM3 cells in the fibrin scaffolds has not been reported so far, the cause of this event cannot be withdrawn from the literature and should be investigated in the future studies.

The effects of green light irradiation emitted by LED also decreased the cell proliferation with no change in the testosterone secretion. Many studies investigating the effects of light on the proliferation and differentiation of different cell lines including umbilical cord mesenchymal cells¹¹, dental pulp cells²¹ and bone marrow mesenchymal stem cells¹⁷ have resulted in conflicting results. Whether such inconsistent results are due to the differences in the type of light emitted, the duration of the radiation, the length of the light treatment period, or even the sensitivity of cell types to light exposure requires further investigations. However, Niu et al (2015) concluded that red and blue light together enhance the anti-proliferative effects of curcumin and reduce the proliferation and apoptosis of skin keratinocytes²². Also, Mingyu He et al (2020) showed that blue LED stops cell proliferation, migration and invasion, and ultimately induces apoptosis in human hepatoma cells by damaging their DNA²³. The results of the last two studies on the cell proliferation are in agreement with our results. However, studies on the testosterone secretion in the animals has shown that exposure of animals to different types of light increases the testosterone production²⁴⁻²⁶. In our study we examined a particular wavelength of 530 nm which did not induce any proliferative or steroidogenesis effect on TM3 cells but combination of LED irradiation with fibrin scaffold increased the proliferative capacity and testosterone production in TM3

cells. Whether steroidogenesis phenomena in the cell machinery could be influenced by different light wavelengths is not clear and the current knowledge could insufficiently provide a precise answer.

According to our result, supplementation of culture medium with melatonin, which has received a great deal of attention in the recent years, significantly reduced proliferation of TM3 Leydig cells and also decreased testosterone secretion. This decrease was also observed in the expression of the relevant genes. Similar to the results of the present study, S Valenti et al (1999), found that melatonin reduced the testosterone secretion of rat Leydig cells through new mechanisms²⁷. Jian Tong et al (2015) also concluded that melatonin inhibits testosterone synthesis in TM3 Leydig cells by inhibiting the expression of SF1 and GATA4 genes²⁸. In contrast to the previous studies, in 2018 Deng et al reported that melatonin increases testosterone secretion in Leydig cell when were concurrently cultured with Sertoli cells, as well as increase in the expression of ROR α gene²⁹. Another study on a Leydig cancer cell line showed that suppression of melatonin receptors, MT1 and MT2, stopped the genes in the testosterone synthesis pathway³⁰. These conflicting results require closer attention especially considering that in our study when melatonin was concurrently administered with fibrin scaffold and LED green light, the genes involved in proliferation and also the genes involved in steroidogenesis were highly expressed; concluding that a 3D system would have lessened the inhibitory effects of melatonin on cell proliferation and testosterone production.

Conclusion

We may conclude that the use of three-dimensional fibrin scaffold has a significant role in cell proliferation, testosterone secretion and the expression of the genes related to the testosterone synthesis pathway; GATA4 and ROR α , in TM3 cells. In contrast, melatonin has a reducing role in Leydig cell proliferation and testosterone secretion. Although visible light radiation did not cause noticeable changes on cell proliferation and testosterone secretion, but in some cases caused a significant decrease. Light irradiation accompanied with a fibrin scaffold follows the additive effects of scaffold on the two mentioned processes and with melatonin follows the decreasing effects of melatonin. On the other hand, the simultaneous use of three variables could significantly mitigate the decreasing effect of melatonin and the overall result was an increase in the cell proliferation and testosterone secretion. Before the findings of the present study could be used for the treatment of patients suffering from low androgen levels, further studies on the different scaffolds and LED irradiation to promote testosterone secretion is needed.

Materials And Methods

Chemicals and reagents

The research was approved by the ethical committee of Kerman University of Medical Sciences with the Ethics Approval Code: IrKMU REC: 1397-135.

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, USA). Fetal bovine serum (FBS) and Penicillin streptomycin were purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd (Hangzhou, China). Mouse Leydig (TM3) cell line was purchased from Pasteur Institute (Tehran, Iran). Mouse Testosterone ELISA Kit was purchased from ZellBio GmbH/Germany. RNX plus solution (Cinnagen, Iran). cDNA synthesis kit and all reverse transcription (RT) substances were purchased from Yektatajhez Company (Tehran, Iran). SYBR Green I Master was purchased from Genaxxon bioscience (Ulm, Germany). hCG was purchased from Merck/Germany. Thrombin was provided as a generous gift by Dr M. Farsinejad from department of Hematology, Faculty of Paramedicine, Kerman University of Medical Science, Kerman, Iran. Other chemicals used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis. MO. USA).

Cell culture and experimental groups

TM3 cells were cultured in DMEM-F12 supplemented with 10% FBS, 1% penicillin & streptomycin and 1% amphotericin (complete culture medium) at 37°C with 5% CO₂ in the atmosphere. After reaching 80% confluence, the cells were harvested by trypsinization and different treatments were enrolled according to the following groups (table 1).

2D culture

The harvested cells were cultured at 1×10^4 in 96 well culture plates for 24 h in DMEM-F12 supplemented with 10% FBS and 1% antibiotics at 37°C with 5% CO₂ in the humidified atmosphere as 2D control group. Some wells received 10^{-7} M melatonin as MEL group.

Fibrin 3D culture

Ten μ l of fibrinogen extracted from human plasma, 10 μ l cell suspension (1×10^4) in PBS and 10 μ l thrombin were transferred into a 1.5 ml microtube and mixed thoroughly. The samples were then incubated in a 37°C incubator with 5% CO₂ in the air for 10 min. After fibrin clot formation, it was pushed out of microtube and transferred into 96 well culture plate (3D, SC group). 10^{-7} M melatonin was added to some wells 24 h later as SC+MEL group.

LED irradiation

A handmade LED device with green light emission was used in the LED exposure groups (LED, MEL+LED, SC+LED and LED+SC+MEL). Twenty-four hr later the medium was refreshed and the samples were once irradiated for 10 minutes at radiation energy of 1.5 J/cm², and 530 nm wavelength ³¹. All the exposure procedures were carried out inside a 37°C CO₂ incubator. In the MEL+LED group the cells/scaffolds were treated with 10^{-7} M melatonin and were then irradiated as explained above.

TM3 proliferation assessment

MTT assay was used to estimate the proliferation of TM3 cells. Briefly, cell density was adjusted to 7500 cells per well/ fibrin scaffold in 96-well plates. After different treatments, the cells/scaffolds were incubated for 72 hr at 37 °C under 5% CO₂ in the air. Twenty µl MTT solution (5 mg/ml) was added into each well and incubation was continued for 4 hr. The supernatant was discarded afterward and 150 µl DMSO was added into each well. The absorbance of each well was measured at 490 nm against a reference wavelength of 630 nm. All the experiments were done in triplicates ³².

Cell viability assessment

3×10⁴ TM3 cells were cultured in the 24 well plates with 300 µl complete culture medium. After 72 hr of the different treatments, the supernatants containing floating cells were transferred into 1.5 ml microtubes. The attached cells were washed with PBS, detached by trypsin EDTA solution, and added to the microtubes with gently pipetting. Ten µl of the cell suspension was mixed with 10 µl of trypan blue, loaded onto a Neubauer chamber and the number of viable and dead cells were counted in 4 large square under an inverted microscope. This assessment was not applicable for the scaffold-containing groups.

The cell viability rate was assessed using the following formula: cell viability rate = number of viable cells/total cell number

Testosterone assessment

The level of testosterone in the supernatant was assessed using an ELISA kit was purchased from ZellBio GmbH (Germany). In brief, 3×10⁴ viable TM3 cells were cultured in 24 well plates with complete culture medium for 24 hr, and different treatments were applied according to the study groups. 48 hr later, 10 IU/ml hCG was added to each well, and 24 h later the supernatant was removed and stored at -20 °C for testosterone measurement. The testosterone level was evaluated according to the protocol provided by the manufacturer ³³.

RNA extraction and cDNA synthesis

Total RNA was isolated using RNX plus (1 ml) and chloroform (200 µl) solutions at room temperature. After centrifugation at 12000 rpm at 4°C for 15 min, the aqueous phase was replaced into a new microtube, cold isopropanol solution was added and was then gently inverted 10 times. The tubes were transferred to a -20 °C freezer overnight. The next day, after centrifugation at 12000 rpm (4°C) for 10 min, one ml 75% ethanol was rinsed on the pellet, the tubes were centrifuged at 7500 rpm (4°C) for 5 min, the ethanol was removed and the pellet was dissolved in diethyl pyrocarbonate (DEPC) water and stored at -20 °C. The concentration and purity of the isolated RNA were assessed by absorbance readings on a UV spectrophotometer (Thermo Scientific™ NanoDrop 2000) at the wavelengths of 260 and 280 nm to obtain a ratio of 1.81 ± 0.06. The RNA integrity was then determined by 1% agarose gel electrophoresis. The cDNA synthesis was performed using cDNA synthesis kit (Yektatajhez azma, Tehran, Iran) according to the manufacturer's protocol: 70 °C for 5 min (RNA denaturing), 42°C for 60 min (cDNA synthesis) and then 70 °C for 10 min (enzyme inactivation).

Quantitative real time PCR

Quantitative real time PCR was carried out to evaluate the mRNA expression level of *PCNA*, *CYCLIND1*, *CDC2*, *CDKN1B*, *GATA4* and *RORα* genes in the TM3 cells by using magnetic induction cyclers (mic) Real-time PCR system (Australia). For the amplification reactions, the synthesized cDNA solution (1 μl) was mixed with specific primers (1 μl) and SYBR Green I Master (10 μl) at a total volume of 20 μl based on the following PCR program (table 2). Primer sequences are described in Table 3. *GAPDH* was used as a housekeeping gene to normalize the qRT-PCR reactions. The $2^{-\Delta\Delta CT}$ method was used to measure gene expression levels.

Declarations

Conflict of interest: The authors do not have any conflict of interest to declare.

Contributions: B. Alavi, designed the study and carried out the laboratory activities; M. Shojaei, prepared the LED device, supervised LED irradiation part of the study and prepared the manuscript; T. haghpanah, designed the study and supervised the molecular and statistical tests; V. Mirzaei, interpreted the data and prepared the manuscript; M. Abedini esfahlani, designed the study and carried out some laboratory activities; M. Jalalkamali, carried out some irradiation procedure and helped in manuscript preparation; F. Seyedi, designed the study and supervised scaffold preparations; SN. Nematollahi-mahani, designed and supervised the study, provided funding and prepared the manuscript.

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Tables

Due to technical limitations, table 1,2 is only available as a download in the Supplemental Files section.

Table 3: primers sequences

Genes	Primer sequences	Accession number
<i>GAPDH</i>	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTCA	- NM_001289726.1
<i>Proliferating cell Nuclear antigen (PCNA)</i>	Forward: CTCCGAAGGCTTCGACACA Reverse: ATTTTGGACATGCTGGTGAGGT	- NM_011045.2
<i>Cyclin D1</i>	Forward: GATGCTGGAGGTCTGTGAGG Reverse: TTCTCGGCAGTCAAGGGAAT	NM_007631.2
<i>CDC2</i>	Forward: CCCGGCGAGTTCTTCACAG Reverse: CAGCGTCACTACCTCGTGTG	- NM-007659
<i>Cyclin-dependent kinase inhibitor (CYCLIN D1) (CDKN1B)</i>	Forward: ACGGGAGCCCTAGCCTGGAGC Reverse: TGCCCTTCTCCACCTCTTGCC	- NM_009875.4
<i>RAR-related orphan receptor alpha (RORα)</i>	Forward: CGCAGCGATGAAAGCTCAAAT Reverse: CAGGAGTAGGTGGCATTGCT	- NM_001289916.1
<i>GATA-4</i>	Forward: GAAGACACCCCAATCTCGTAGA Reverse: TAATGAGGGGCCGGTTGATG	- NM_001310610.1

Figures

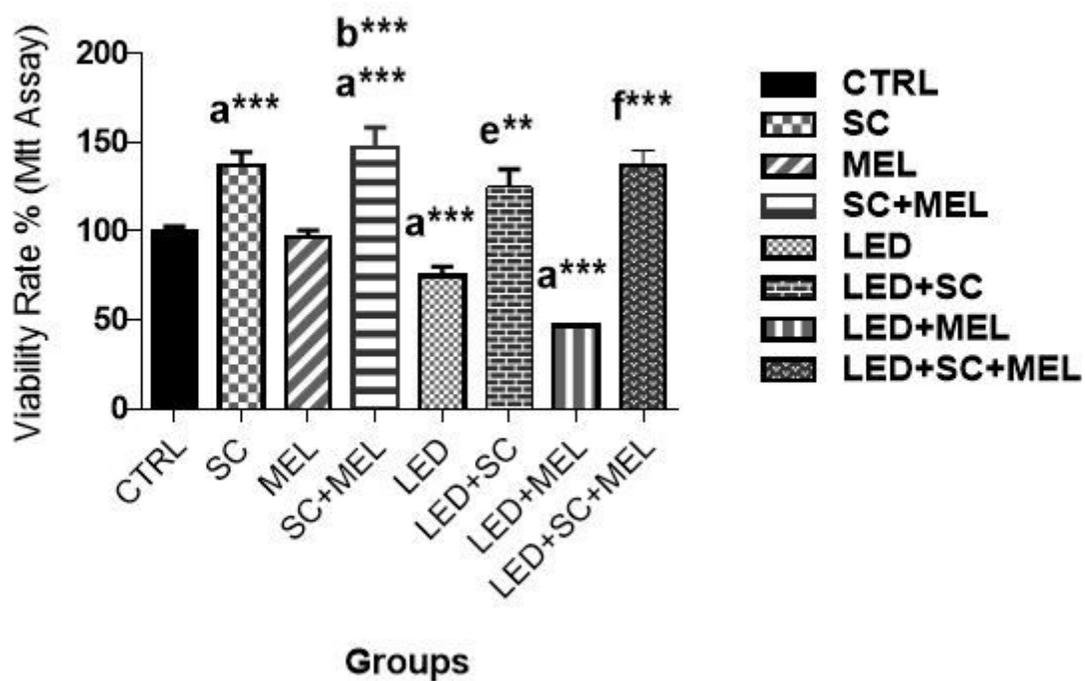


Figure 1

Cell proliferation measured by MTT assay in the different groups. Values are the mean \pm SE. a) significant difference compared to CTRL group. b) significant difference compared to MEL group. e) significant difference compared to LED group. f) significant difference compared with LED + MEL group. ** $P < 0.01$; *** $P < 0.001$. CTRL: control Mel: melatonin LED: light irradiation SC: scaffold

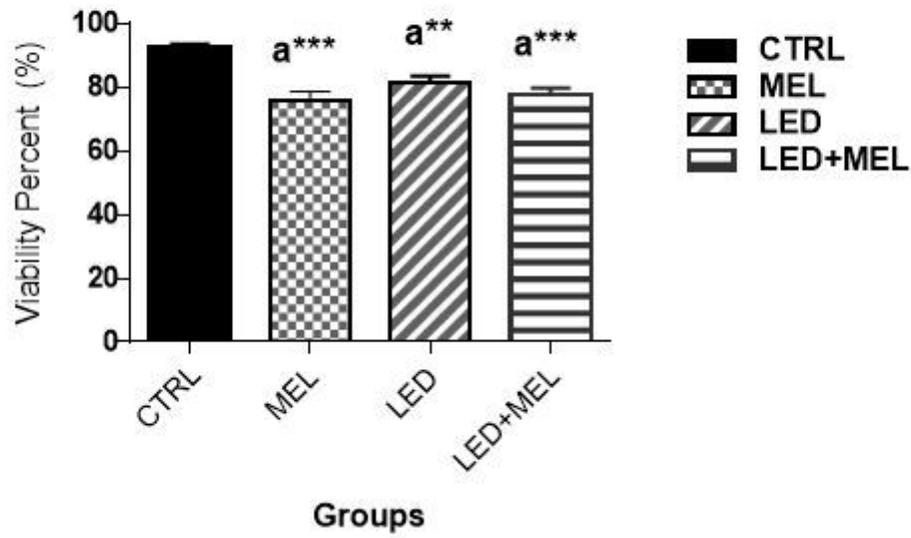


Figure 2

Viability rate of the TM3 cells in the different treatment groups using trypan blue staining. Values are the mean \pm SE. a) significant difference compared with CTRL group (**P < 0.01; ***P < 0.001). CTRL: control MEL: melatonin LED: light irradiation

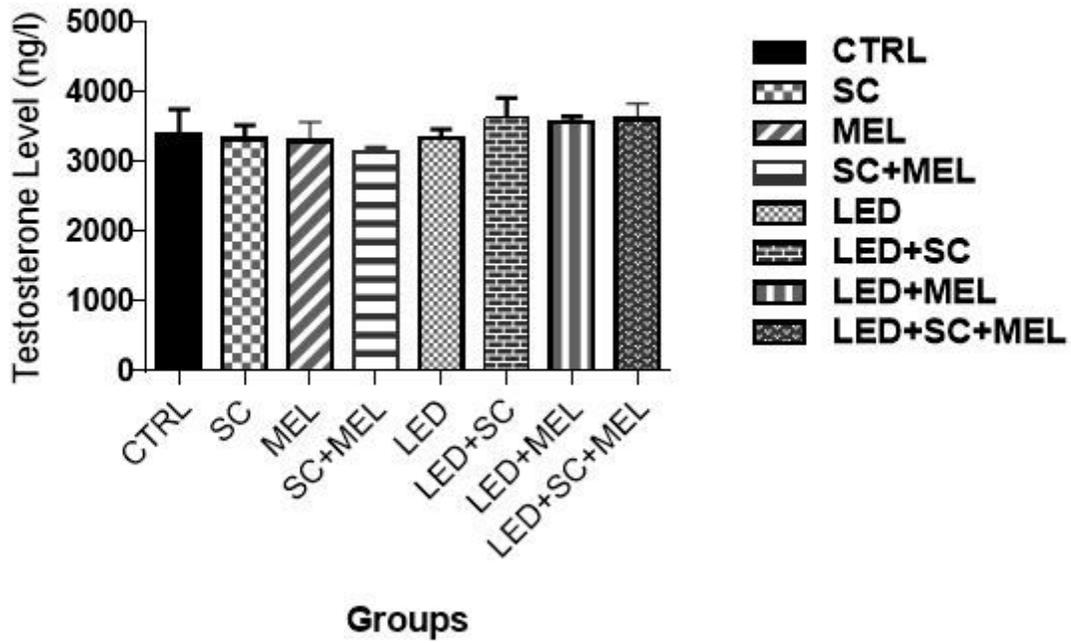


Figure 3

The testosterone level in the different groups 24 hr after a hCG challenge test 18. CTRL: control MEL: melatonin LED: light irradiation SC: scaffold

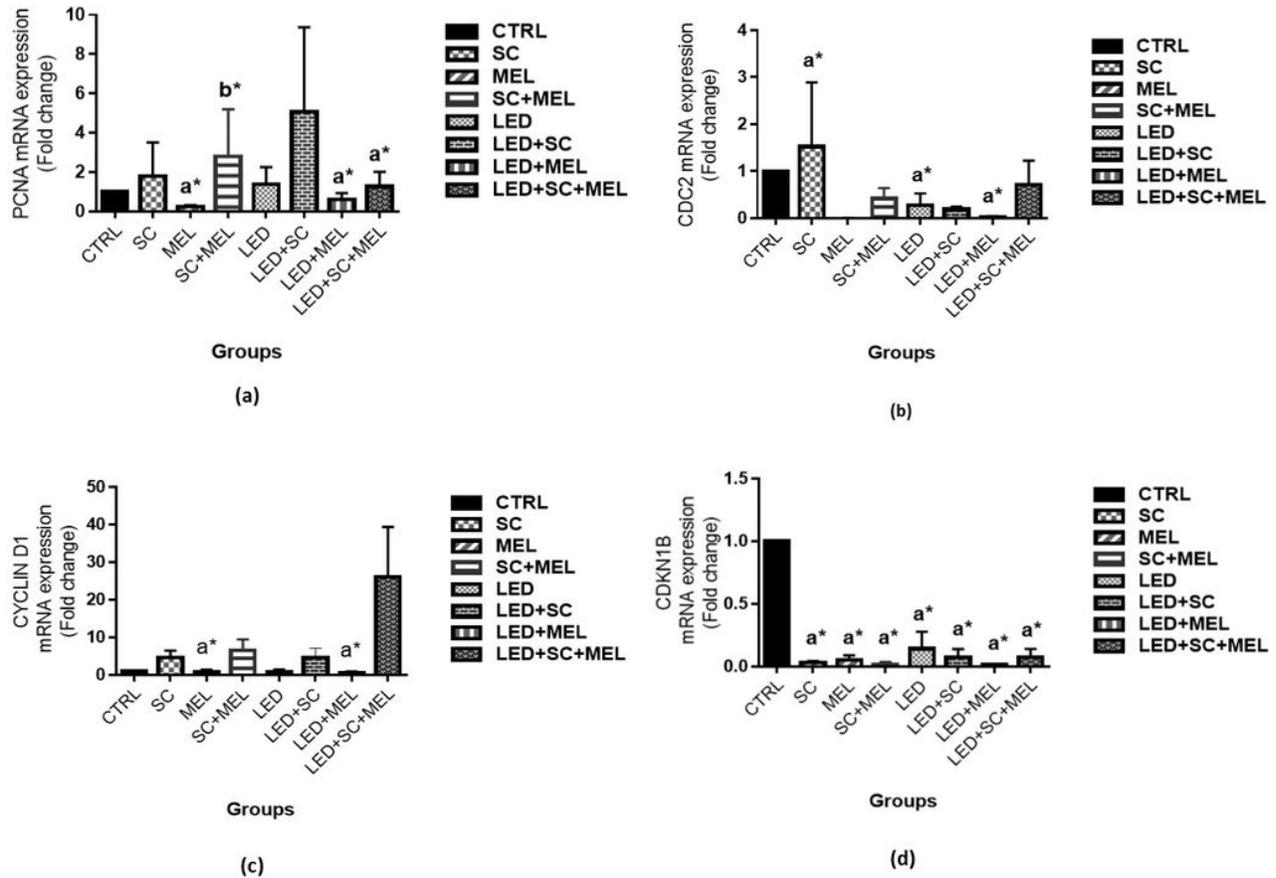


Figure 4

a. The expression pattern of PCNA gene in the different groups. b. The expression pattern of CDC2 gene in the different groups. c. The expression pattern of CYCLIN D1 gene in the different groups. d. The expression of the CDKN1B gene in the different groups. a) significant difference compared with CTRL group. b) significant difference compared with MEL group. *, P<0.05). CTRL: control MEL: melatonin LED: light irradiation SC: scaffold

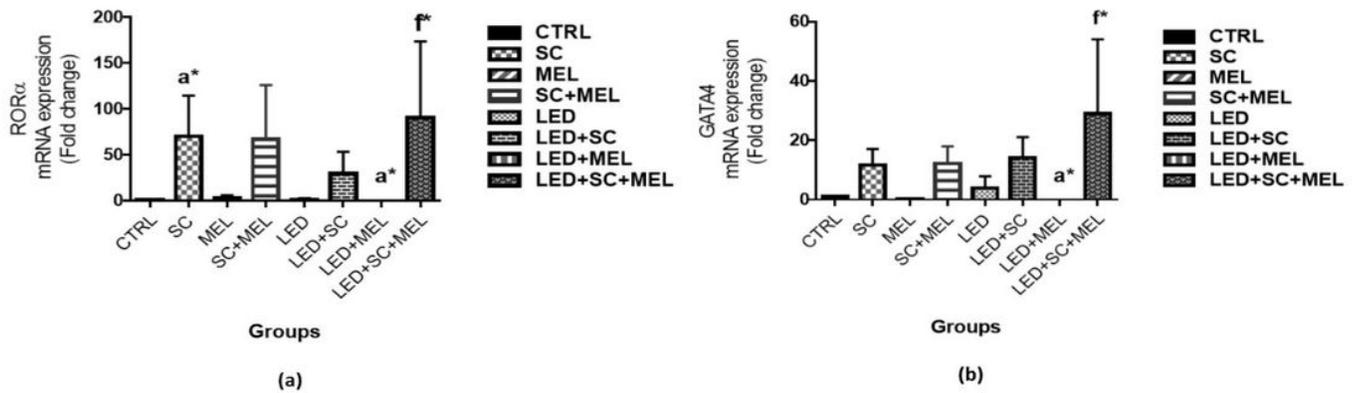


Figure 5

a, b. The expression of the ROR α and GATA4 genes in the different groups. A, significant difference compared with CTRL group. F, significant difference compared with LED+ MEL group (*P < 0.05). CTRL: control MEL: melatonin LED: light irradiation SC: scaffold

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.jpg](#)
- [Table2.jpg](#)