Endogenous Melatonin Concentration Along With the Expression of Mitochondrial Regulator Genes Is Elevated by the Serum Shock Process in the U87-MG Cell Line

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Abstract

Glioblastoma, also known as the fourth grade in the development of astrocytoma according to the World Health Organization, is a tumor in the glial region confined to the central nervous system with high invasion capability to the parenchyma of the brain. Recent findings suggest that melatonin can be synthesized outside the pineal gland tissue. Mitochondria can produce melatonin independently but in coordination with cell demands which plays a critical role in regulating the cell cycle and cell metabolism. hence, we aimed to examine the relationship between cell metabolism and the induction of endogenous melatonin increase induced by the serum shock process, then, determine the percentage of cell proliferation.

Background: glioblastoma is a highly invasive tumor of glial cell of brain tissue. Recently it was reported that melatonin can be produced in mitochondria organelle of the glioblastoma cells independent to pineal gland. Regarding the physiological function of melatonin released from pineal gland in regulation of rhythmicity, here we aimed to investigate if serum shock standard protocol known for cellular rhythm regulator can change the melatonin production ability of the glioblastoma cell.

Material and methods: First, U87-MG glioblastoma cells were cultured in a DMEM medium containing 10% FBS and then cells were treated with a standard serum shock process (no FBS, 8h). The concentration of melatonin was measured using ELISA method in supernatant and cell extracts of Shock and control groups. The cell proliferation was measured by using BrdU staining and flow cytometry assessment. The gene expression levels of some mitochondria or circadian related genes including TFAM, BMAL1, PGC-1α, and DRP1 were measured, using qRT-PCR method.

Results: our findings showed increased (two times) concentration of cellular and released endogenous melatonin in the FBS shock treated U87-MG glioblastoma cells compared to the control group. we found significant up-regulation of the mitochondria or circadian regulator genes (TFAM, BMAL1, PGC-1α, and DRP1) at mRNA level; in the FBS shock group compared to the control group ($P < 0.0002$). Moreover, the percent of proliferative cell (Brdu positive) was also elevated in FBS shock group however it was not statistically significant.

Conclusion: the serum shock process has a far effect on the cellular behavior of the U87-MG cell line. regard to the results of the study, it is worth mentioning that an increase in the concentration of endogenous melatonin affects many signaling pathways within the U87-MG cell line, and the elevated expression of the candidate genes was the proof of this fact.

by considering the results of this study it also should be noted that detailed investigating the role of endogenous melatonin and its effects on cancer cells is pivotal and by comparing the results of the normal cells with cancer cells we can find the hotspots of the involved signaling pathways that could help better understanding the biology of glioblastoma.

Introduction:

Glioblastoma is the most common type of primary malignant brain tumor and causes the highest mortality among patients with primary brain tumors. Although considerable advances have been made in understanding the biology of this tumor, but it still has poor prognosis and more investigation is appealing to help novel therapeutic methods. Changes in the tumor metabolism are fundamental events in the process of transformation and adaptation to tumor micro environment. Studies have previously shown that glioblastoma cells use mitochondrial respiration to achieve energy homeostasis under starvation conditions.[1–2] In previous studies, the focus has been on the effects of exogenous melatonin injection as an effective treatment for cancer regardless of the fact that the cancer cells themselves have a melatonin-based homeostasis system that specifically helps them to adapt to changes. Melatonin (N-acetyl-5-methoxytryptamine), a hormone secreted by the pineal gland, was first isolated from the bovine pineal gland in 1958. this hormone is secreted by the pineal gland of most vertebrates including humans; therefore, the pineal gland is considered to be the main site of melatonin synthesis. However, Melatonin can also be synthesized in the mitochondria of the other brain cells. Given that mitochondria are the main site of free radical production in the cell, the synthesized melatonin can function as a potent endogenous free radicals scavenger to protect cell from oxidative stress.[3–5]

Previous studies have reported that melatonin treatment can decrease the expression of TFAM and other mitochondrial transcription factors like TFB1M and TFB2M in association with decreased expression of mitochondrial NADH dehydrogenase 1 gene. Alterations in the expression of mitochondrial genes or nuclear genes can cause a decrease in the mitochondrial respiratory chain to increase ROS production, which in turn may trigger apoptosis.[6–7]

Circadian rhythm disturbances, affecting the CLOCK/BMAL1 system, are involved in the expression of the aryl alkylamine N-acetyltransferase (AANAT) gene. It is worth mentioning that this enzyme is one of the essential factors in the synthesis of melatonin, which plays a role in the conversion of serotonin to melatonin. On the other hand, through proteasome inhibition, melatonin has been shown to maintain a significant amount of BMAL1 in cells. Recognition of BMAL1 as a tumor suppressor in most cancer cells enhances the protective role of melatonin.[8–9]

It is important to note that control of mitochondrial dynamics is essential for cellular and tissue bioenergetics. One of the most important genes involved in this pathway is the DRP1 gene. Studies on Parkinson's model cells have shown that the neuroprotective effect of melatonin is mediated by inhibition of oxidative stress and DRP1-dependent mitochondrial fragmentation.[10–11]

One of the most important mediators of metabolic adaptation is PGC1α. Metabolic and mitochondrial genes have shown increased expression in parallel with high PGC1α expression. For example, the TCA cycle, OXPHOS, lipogenesis, and antioxidant genes were highly expressed in cells with high levels of PGC1α. These findings indicate a close relationship between PGC1α gene expression and energy metabolism in glioblastoma cells. It also demonstrates the fact that changes in PGC1α expression can be very vital in the proliferation of the Glioblastoma cancer line.[12–14]

In previous studies, the focus has been on the effects of exogenous melatonin injection as an effective treatment for cancer; regardless of the fact that the cancer cells themselves have a melatonin-based homeostasis system that specifically helps them to adapt to changes. These findings, along with the results of other studies, led us to investigate one of the possible pathways of melatonin in the cell. For this purpose, it was necessary to create conditions for cellular metabolic stimulation and cellular rhythm readjustment in order to increase the concentration of melatonin.[4]

Materials And Methods:

Cell Culture: The human glioblastoma cell line U87-MG (NCBI Code: C531) was purchased from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran). The cell line was grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA) and supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA) and also Penicillin-Streptomycin antibiotic (GIBCO, USA). The cells were then incubated at 37°C in a humidified atmosphere of 5% CO2 environment.

Serum shock process: To perform this method, U87-MG cells with an approximate number of 3×105 cells per well were seeded in 6-well culture plates and placed in a cell culture incubator for 24 hours. when the seeded cells reached to 80% confluency, the timed process of serum shock was begun.

The number of 3 of 6 existing wells were marked as FBS shock group and the 3 others were considered as the control group; At time = 0, the medium of shock group was exchanged with serum-rich medium (DMEM + pen strep, supplemented with 50% horse serum [Bahar Afshan, Iran]), In this 2-hours interval, the cells of the control group were treated with a medium containing 10% of FBS serum. then, both groups were incubated for 8 hours with serum-free DMEM + pen strep. At the end of the 8-hours period, the cell Supernatant was carefully collected and centrifuged in ribonuclease free micro tubes and placed at minus 70 ° C freezer to be used to measure melatonin. Finally, after the trypsinization process for cell harvesting, cell precipitate was stored in the minus 70 ° C freezer to measure intracellular melatonin, and extract total RNA for studying genes expression.

Melatonin assay with ELISA kit: Melatonin assay was performed on both cell supernatant and cell lysates by (Elabscience Human Melatonin ELISA Kit; lot no: 1RDJVQ8SSG). First Added 50µL standard or sample to each well. Immediately added 50µL Biotinylated Detection Ab to each well. Micro plate was Incubated for 45 min at 37°C. then removed and washed 3 times. after that 100µL HRP Conjugate was Added to each well and incubated for 30 min at 37°C. again, Aspirate and wash 5 times. in the next step 90µL Substrate Reagent was Added and Incubate 15 min at 37°C. at the end 50µL Stop Solution was added and read at 450nm immediately. The concentration of human melatonin in the samples was then determined by comparing the OD of the samples to the standard curve. The results were expressed in Pg/mL. According the manufacturer the Sensitivity or the minimum detectable dose of Human Melatonin was 9.38 Pg/mL and the Detection range of method was 15.63–1000 Pg/mL.

Cell Proliferation Assay by Flow Cytometry: At first, U87-MG cells that had been in the cell culture flask for 24 hours was harvested and seeded to a 6 well cell culture plate. Then the mentioned protocol for serum shock was induced on the cells with a brdu supplementation during the 8 h incubation time in serum free medium. At the end of 2 hours of shock, all the culture medium was replaced with medium containing BrdU (3mM) in a volume of 1 ml of basic culture medium (serum free). After 8 hours and exposure of the cells to BrdU during this time, both cell groups (shock and control groups) were harvested and washed with cold PBS, and centrifuged (At 4 ° C for 3 minutes at 3000 rpm). And then cells were permeabilized and denaturated using 2 N HCL and Triton 100X (0.5) for 30 minutes at room temperature. following 3 times wash with the cold PBS; the cells were stained with anti-BrdU antibody (Monoclonal FITC Mouse IgG1, κ Isotype Ctrl (ICFC) (Bio Legend) with tween20 (0.5%) and BSA (1%) for 90 minutes in the dark. In the last step, the cells were washed 3 times with cold PBS and the proliferation rate was measured by flow cytometry at Blue Laser (488 nm).

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR):

Total RNA of the cells was isolated with by RNx Plus extraction kit (SINACLON, Tehran, Iran) and reverse transcribed into cDNA using the cDNA synthesis Kit (YTA, Tehran, Iran). Transcript levels were determined by real-time qRT-PCR using SYBR Green qPCR master mix 2x (100 rxn-Antibody base) (YTA, Tehran, Iran) and performed in a Real-Time PCR Thermal Cycler. The optimal primers for PCR were as follows:

Primer Name	Sequences (5'→3')
GAPDH	**Forward: 5'- ACAGTCAGCCGCATCTTC -3'**
GeneID: 2597	**Reverse: 5'- CTCCGACCTTCACCTTCC -3**
Human PGC-1α	**Forward: 5'- CCAAAGGATGCGCTCTCGTTCA – 3'**
GeneID:10891	**Reverse: 5'- CGGTGTCTGTAGTGGCTTGACT – 3'**
TFAM	**Forward: 5'- CCGAGGTGGTTTTCATCTGT – 3'**
GeneID: 7019	**Reverse: 5'- GCATCTGGGTTCTGAGCTTT – 3'**
BMAL-1	**Forward: 5'- CAGCCAGTGATGTCTCAAGC – 3'**
GeneID: 406	**Reverse: 5'- ATGCGTGTCCGTTGTTCC – 3'**
Drp1	**Forward: 5'- GATGCCATAGTTGAAGTGGTGAC – 3'**
GeneID: 10059	**Reverse: 5'- CCACAAGCATCAGCAAAGTCTGG – 3'**

each amplification was performed in triplicate, and expression levels were calculated using the 2 − ΔΔCT method, with GAPDH serving as the normalization control.

Results:

Effect of serum shock on U87-MG cell line morphology and proliferation

Serum shock can affect the morphology and proliferation rate of U87-MG cancer cells Preliminarily the morphological changes of the treated cells were monitored using light microscopy. Expectedly we found different morphological difference in shock group with more elongated oval shaped cells with stretched out cellular extensions however the control group cells showed more circular shape with obviously less extensions (Fig. 1) after 8h of serum free incubation. The 2h high serum concentration treatment in shock group can help cells to tolerate the 8 h of serum free condition compared to control group. Since the standard serum shock protocol used in current study was previously reported to have fundamental effect in cellular metabolic regulation and also circadian activation[22], so far, the observed morphological change could be explained consequently.

To investigate the effect of serum shock in proliferation rate of the U87-MG glioblastoma cancer cells, the proliferative Brdu positive cells were measured using flowcytometry. Our findings showed increase in proliferation rated in shock treated group, however it was not significant (Fig. 2)

Serum shock treatment increase the melatonin concentration in U87-MG glioblastoma cells

in order to investigate the effect of serum shock in melatonin production (content) and release in U87-MG glioblastoma cells, the intracellular and released melatonin concentration in culture medium were measured using ELISA method. Our findings showed significant elevation of melatonin concentration in cell culture supernatant approximately > 3 times with 10+-SE (Pg /ml) in control vs 35+-SE (Pg /ml) in shock group ($P < 0.0003$) (Fig. 2A).

Furthermore, as shown in Fig. 2B, in the cell lysate the level of measured melatonin was measured higher than supernatant (~ > 10 times) and again the shock group U87-MG cells showed significant ($P < 0.0003$) increased level of melatonin (500+-SE Pg/ml) in comparison to control (200+-SE Pg /ml).

Serum shock treatment increase the mitochondrial regulator genes expression in U87-MG glioblastoma cells: As was expected, the gene expression of the Bmal-1 as one of the main circadian regulators was significantly ($P < 0.0002$) upregulated (2+-SE folds increase) in the serum shock treated cells, indicating circadian pathway activation.

The gene expression experiments showed significant increase of mitochondria transcription factor gene, TFAM (2.5+-SE folds increase, $P < 0.0002$) in serum shock group. Also, the gene expression of the key mitochondrial fission regulator, DRP1 was upregulated in the serum shock treated U87-MG cells in comparison to control, (2+-SE folds increase, $P < 0.0002$). Moreover, the gene expression of the PGC1α transcriptional coactivator that is a central inducer of mitochondrial biogenesis was significantly elevated in serum shock U87-MG cells up to 2 folds of gene expression ($P < 0.0002$). The fact that serum shock can significantly change of the mitochondrion regulator genes including (TFAM, PGC1α and DRP1) indicates potential effect of serum shock in mitochondrial dynamic in response to metabolic effect of serum shock/starvation treatment.

Discussion:

The serum shock process is one of the approved methods to reset cell rhythm, which regulates cell metabolism and circadian rhythm[17]. In our study, serum shock process was considered as the main variable for rearrangement of cellular metabolism. after the serum shock process and exclusion of U87-MG cells from the serum-containing medium for 8 hours, we saw an increase in rate of the proliferation in the shock-group compared to the control group. It is worth mentioning that, in shock group we observed less cellular damages compared to control group hence, it can be said that, the morphological changes confirm the difference between two groups in the rate of proliferation

Studies have shown that astrocyte cells in the cortex of rats and glioma cells are specifically capable of producing melatonin separately from pineal cells. [15] Melatonin synthesized from a tumor glioma, exerts an endocrine anti-proliferative effect; Thus, more aggressive gliomas synthesize or accumulate less melatonin. [16] After serum shock process We measure the concentration of melatonin in cell culture supernatant and cell layset at the same time. The concentration of melatonin in shock group was 2 times higher than control group. This finding may confirm the fact that, in the specific condition after the serum shock the U87-MG cells start to produce the melatonin which is help them to maintain their metabolic hemostasis.

One of the new findings in recent studies about melatonin is the presence of a transporter for this molecule in the cell. PEPT1 / 2 membrane transporter is the candidate transporter of melatonin in the cell. This protein is located both on the cell membrane and mitochondrial membrane, which is the main site of intercellular synthesis of melatonin. The function of this transporter is to regulate the concentration of melatonin both in intercellular and intracellular of the cell.[18]. The other finding of our study, was an approximately 10-time differences in the concentration of melatonin between intercellular and intracellular of the U87-MG cells. By considering our finding, the further studies should be design to prove the existence of this transporter on U87-MG cell lines.

The cellular changes observed after the serum shock process necessitated the measurement of the expression of certain genes that play pivotal roles in cellular pathways.

One of the genes that studied, is the TFAM gene, or mitochondrial transcription factor A. This gene is one of the main controllers of mitochondrial gene transcription and also plays an essential role in the preservation and stability of the mitochondrial genome. previous studies have shown that this gene can be one of the most important targets in the suppression of glioma tumors; Injection of exogenous melatonin into the U87-MG cell line reduced the expression of this gene, which in turn increased the instability in the mitochondrial genome, that eventually led to apoptosis of U87-MG cells.[6] In our study, with increasing endogenous melatonin concentration after serum shock process, the expression of TFAM gene not only did not decrease but also showed a significant increase in the shock group compared to the control group. This increase in expression by considering the role of TFAM in the stability of the

mitochondrial genome can be assumed as one of the factors involved in increasing the rate of proliferation in this study.

Another gene studied, is the PGC1-α gene. This gene is one of the key genes in regulating cell metabolism by regulating mitochondrial metabolic activity. PGC1-α plays a role by affecting the function of mitochondrial transcription factors. On the other hand, increased expression of this gene increases biogenesis in mitochondria. The results of previous studies have shown that in glioblastoma and especially in U87-MG cell line, increased expression of PGC1-α gene is significantly associated with pathogenesis and malignancy.[19] In our study, the expression of PGC1-α gene was significantly increased, which along with the increase in TFAM expression could explain the maintenance the shock group of the U87-MG cell line.

The BMAL1-CLOCK protein complex promotes transcription of the ROR, Rev-erb families, and PGC-1α gene. On the other hand, Rev-erbα can directly participate in the regulation of PGC-1α expression, mitochondrial biogenesis and autophagy. In addition, circadian rhythm fluctuations, modulate mitochondrial dynamics by regulating Drp1 and ATP.[20] These facts prompted us to measure BMAL1 gene expression after the serum shock process. The results obtained from the analysis of BMAL1 gene expression revealed that the expression of this gene also showed a significant increase along with TFAM and PGC-1α genes. This finding suggests that the process of serum shock and subsequent increase in endogenous melatonin concentration can activate different signaling pathways simultaneously.

Studies have shown that excessive increase or inhibition of PGC-1α gene expression impairs biogenesis and mitochondrial dynamics. However, a moderate increase in the expression of the PGC-1α gene can directly control the expression of the DRP1 gene by binding to its promoter and increase its expression, which results in an increase in stability in mitochondrial dynamics.[21] This finding led us to measure the expression of DRP1 gene in our study after observing the increase in PGC-1α gene expression. Observing a significant increase in DRP1 gene expression, revealed that the serum shock process leads to the regeneration of pathways involved in cellular metabolism, in which the role of mitochondria is essential.

in conclusion, it can be said that U87-MG cells after serum shock produce melatonin, simultaneously in cooperation with elevation in the expression of the genes that control the metabolism in mitochondria and also circadian rhythm. The role of endogenous melatonin in the mention pathways should be studied because of the Extensive effect of this molecule in cellular hemostasis. Of course, it is worth noting that further studies to identify involved pathways in normal cells and other cancer cell lines are of great importance.

Declarations:

Authors' contributions

SB carried out the cell experiments, gene expression assays and prepared the manuscript. MSJ, SB and MF contributed in bioinformatics analysis of data. SB, MSJ and NMS contributed in project design. All

authors read and approved the final manuscript

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Not applicable

Competing interests

The authors declare that they have no competing interests

Availability of data and materials

The data of this study is available from the corresponding author on reasonable request

Ethics approval and consent to participate

Not applicable.

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Figures

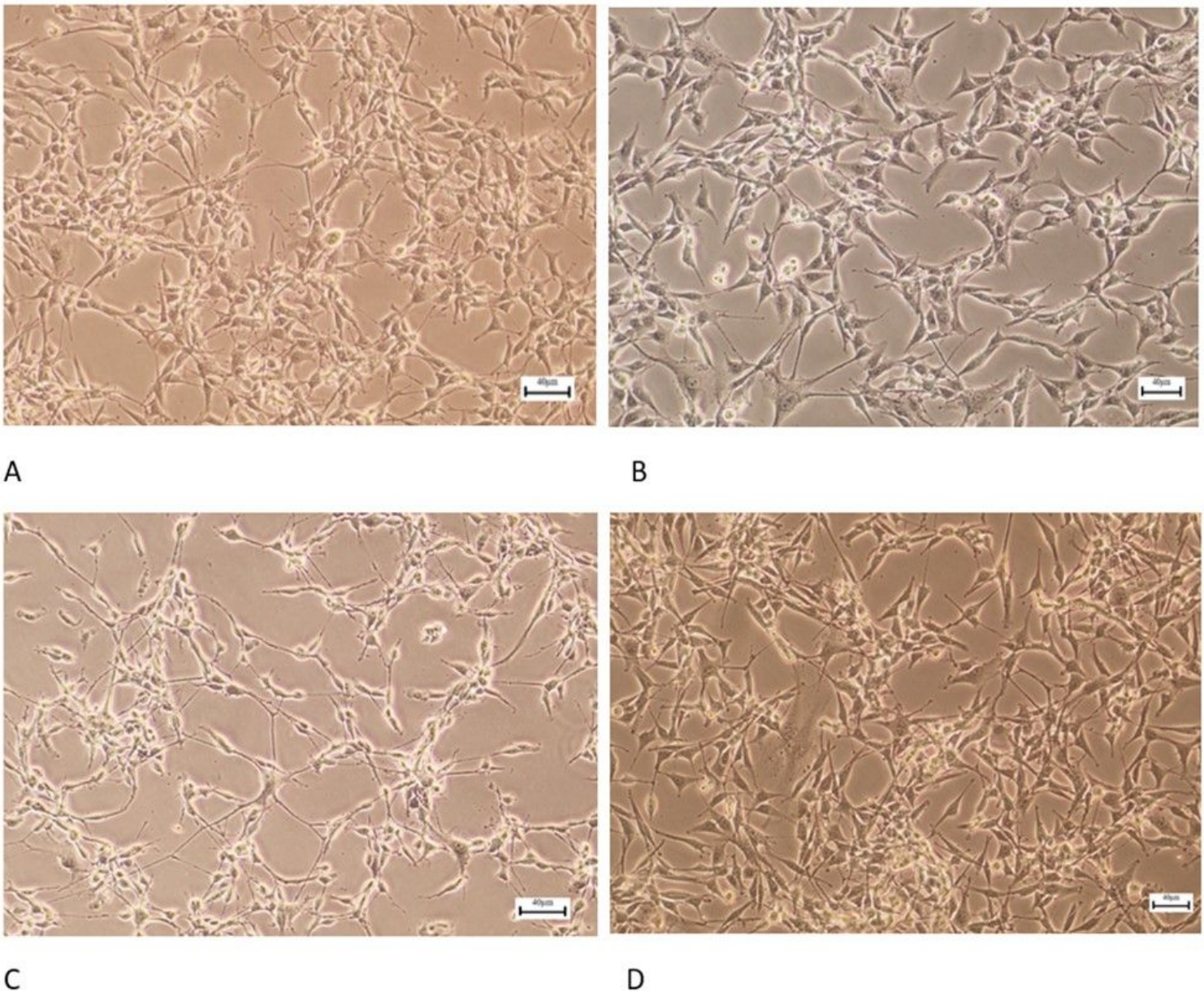

Figure 1

morphological changes of U87-MG glioblastoma cell line in response to serum shock. A) Control group after 2h shcok process with 10% FBS, B) Shock group after 2h shock process with 50% Horse serum, C) Control Group after 8h in serum free medium, D) shock Group after 8h in serum free medium

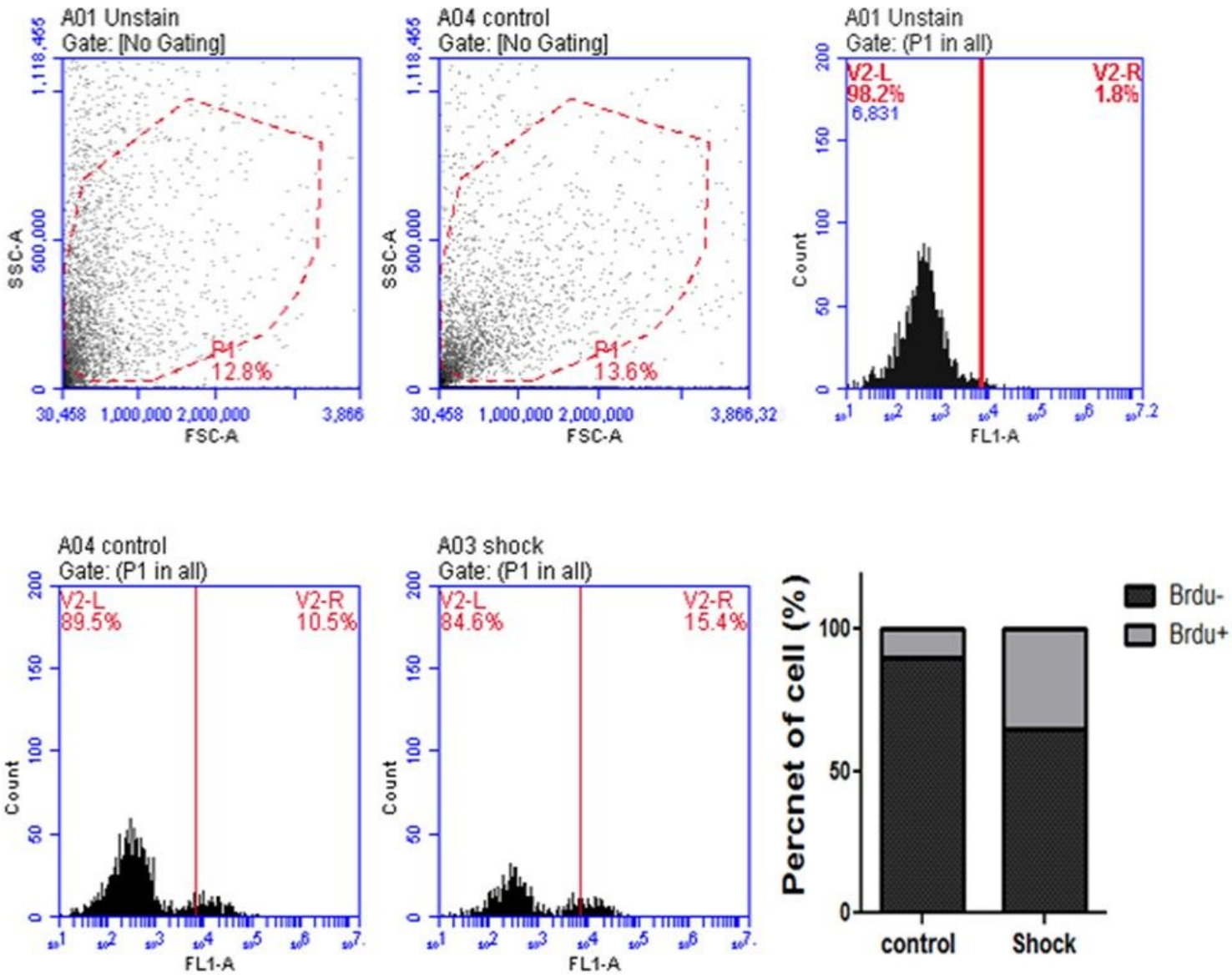

Figure 2

Evaluation of U87-MG cellular proliferation rate by BrdU staining method. The A and B show the forward to side scatter plot of un-stain (A) and control (B) U87-MG cells. The sample histogram plots are shown for un-stain (C), control (D) and serum shock (E) treated U87-MG cells, which Fl-1 indicates BrdU positive cells. The bar chart shows mean of BrdU+ and BrdU- U87-MG cells in each group.

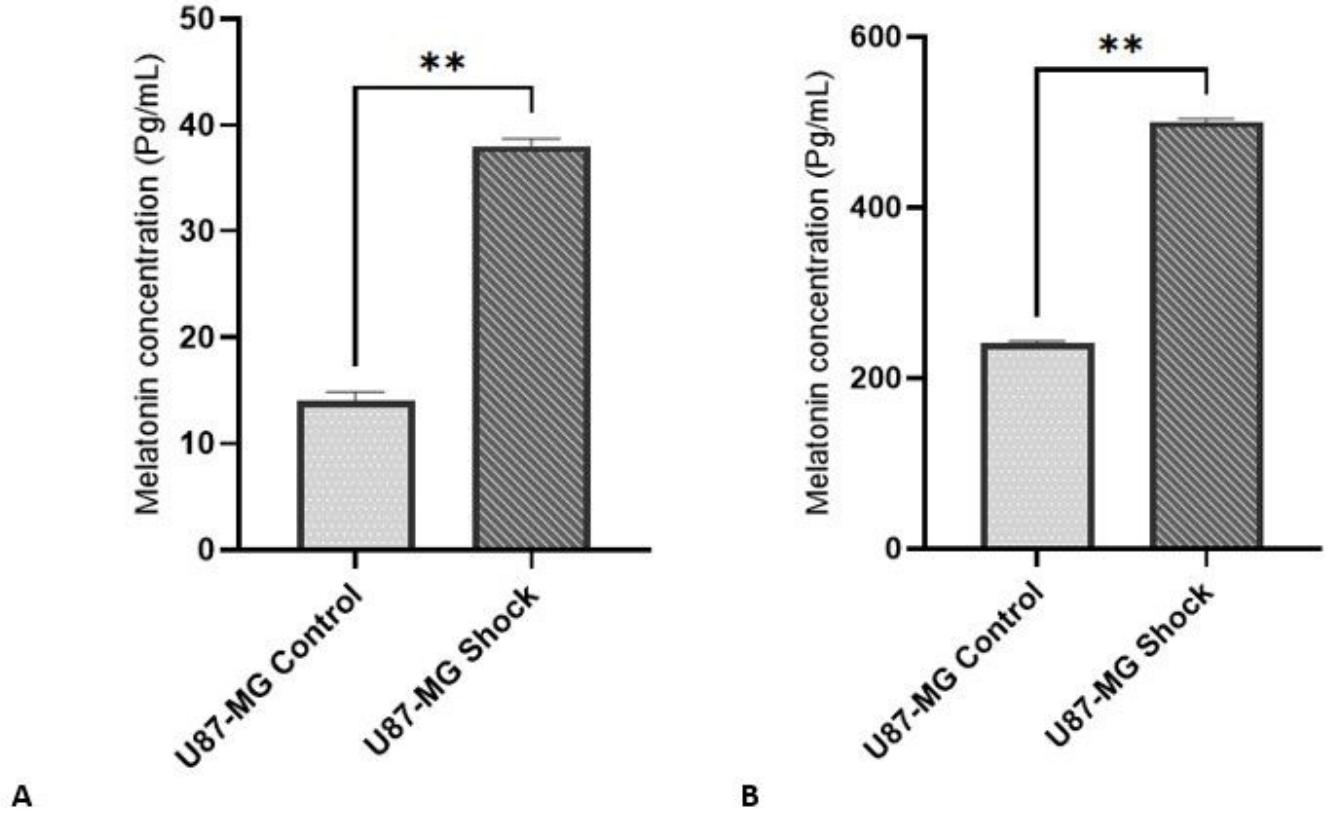

Figure 3

Level of the released (A) and intracellular (B) melatonin concentration in U87-MG cells in response to serum shock.

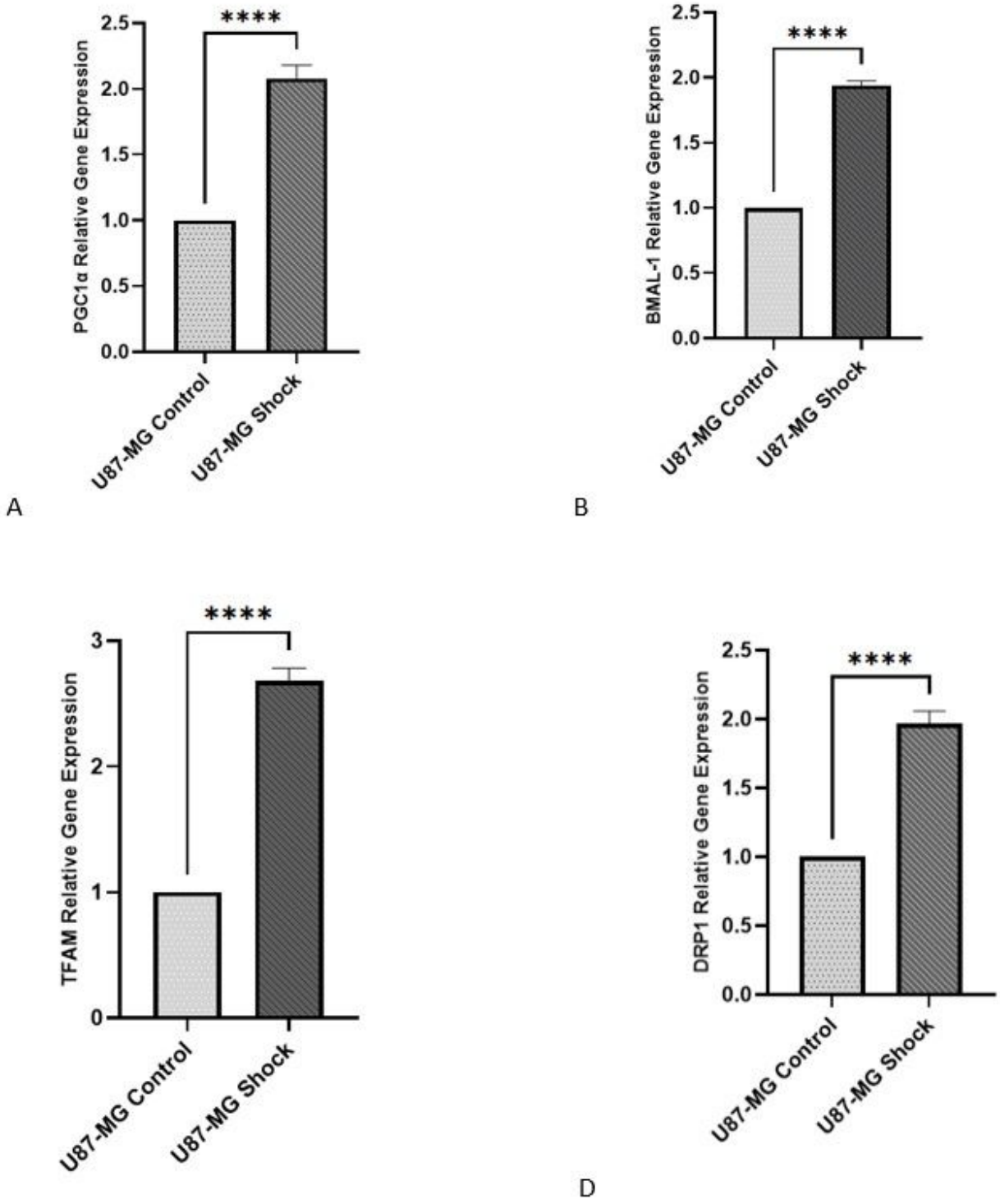

Figure 4

Gene Expressions: Each amplification was performed in triplicate, and expression levels were calculated using the 2−ΔΔCT method, with GAPDH serving as the normalization control. A) the expression of PGC1α gene in U87-MG shock group and control group. B) the expression of BMAL1 gene in U87-MG shock group and control group. C) the expression of TFAM gene in U87-MG shock group and control group. D) the expression of DRP1 gene in U87-MG shock group and control group.