

Original Research

Serum Shock Enhances Endogenous Melatonin Production and Mitochondrial Gene Regulation in U87-MG Glioblastoma Cells

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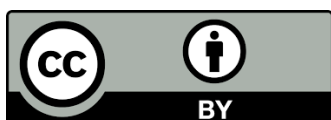
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Abstract

Glioblastoma (GBM) is the most aggressive primary brain tumor, with a poor prognosis despite advancements in understanding its biology. Melatonin, a key regulator of metabolism and cellular homeostasis, is known for its neuroprotective and anti-cancer properties. While traditionally linked to pineal gland secretion, emerging evidence suggests that glioblastoma cells can produce melatonin within their mitochondria. This study investigates whether serum shock can influence endogenous melatonin production and mitochondrial gene expression in glioblastoma cells (U87-MG). U87-MG cells were subjected to serum shock (50% horse serum for 2 hours, followed by serum-free conditions for 8 hours). Melatonin levels in cell supernatants and lysates were quantified using ELISA, while cell proliferation was assessed via BrdU staining and flow cytometry. Additionally, qRT-PCR was used to analyze the



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expression of key mitochondrial and circadian genes (TFAM, BMAL1, PPARGC1A, and DNM1L). Our findings reveal a significant increase in both intracellular and extracellular melatonin levels in serum-shocked cells compared to controls ($p < 0.0003$). Notably, TFAM (Transcription Factor A, Mitochondrial), PPARGC1A (Peroxisome Proliferative Activated Receptor, Gamma, Coactivator 1), BMAL1 (Basic Helix-Loop-Helix ARNT-like 1), and DNM1L (dynamin 1 like) expression levels were significantly upregulated ($p < 0.0002$), suggesting enhanced mitochondrial activity and circadian regulation. Although serum shock slightly increased the proliferation rate of U87-MG cells, the effect was not statistically significant. These results indicate that serum shock triggers metabolic adaptation in glioblastoma cells, leading to increased endogenous melatonin production and enhanced mitochondrial gene expression. This study highlights the potential role of intrinsic melatonin in glioblastoma metabolism, warranting further research into its implications for tumor progression and therapeutic strategies.

Keywords

Serum shock; glioblastoma; melatonin; cell proliferation; mitochondria

1. Introduction

Glioblastoma is the most common and deadliest type of primary brain tumor. Even though researchers have learned much more about how these tumors work in recent years, patient survival rates remain disappointingly low [1, 2]. While some scientists believe that cutting off nutrients might slow tumor growth, the actual experimental data tell a more complicated story. Cancer cells seem to be surprisingly good at adapting and finding ways to survive even in harsh conditions [3, 4].

What makes glioblastoma so challenging to treat is the complex interplay between the tumor and its surroundings in the brain. The cancer doesn't just grow on its own - it interacts with brain chemicals and nerve signals in ways that help it spread. We now know that brain chemicals like serotonin and dopamine play a role in helping the tumor grow [5]. Interestingly, melatonin—the same hormone that regulates our sleep cycles—may have some protective effects against these tumors. Beyond helping with sleep, melatonin appears to fight inflammation, reduce cell death, and protect against damage from harmful molecules [6]. Research suggests that it may be important not only for brain diseases but also for cancer [6, 7]. Interestingly, glioblastoma cells can produce their melatonin within their mitochondria, utterly independent of the pineal gland. We call this "endogenous melatonin." Unlike the melatonin released by the pineal gland, which follows our sleep-wake cycles, this tumor-produced version doesn't exhibit the same daily rhythm. What's particularly intriguing is that while we know cancer cells produce this melatonin, there's no evidence it ever enters the bloodstream like its pineal-derived counterpart does [6, 8, 9].

Research shows that when we give melatonin as a treatment (exogenous melatonin), it can influence essential genes that control cellular rhythms and mitochondrial function. For example, melatonin therapy has been found to dial down the expression of TFAM (Transcription Factor A, Mitochondrial) and other key mitochondrial transcription factors. These changes can disrupt the mitochondrial respiratory chain, creating a cascade effect where cells produce more harmful

reactive oxygen species (ROS) and may ultimately undergo programmed cell death [10, 11]. Melatonin has been shown to keep a considerable quantity of BMAL1(Basic Helix-Loop-Helix ARNT-like 1) in cells by inhibiting the proteasome. The discovery of BMAL1 boosts melatonin's preventive role as a tumor suppressor in most cancer cells [12, 13].

For our cells and tissues to produce energy efficiently, mitochondria need to maintain a careful balance in their structure and function. One of the key players in this process is the DNM1L (dynamin 1 like) gene. Fascinating research using Parkinson's disease models shows how melatonin helps protect nerve cells - it not only reduces harmful oxidative stress but also prevents excessive mitochondrial fragmentation by regulating this DNM1L pathway [14, 15]. PPARGC1A (Peroxisome Proliferative Activated Receptor, Gamma, Coactivator 1) is one of the essential mediators of metabolic adaptation. In conjunction with upregulated PPARGC1A expression, there is a concomitant increase in the expression of genes involved in metabolic and mitochondrial processes. Specifically, elevated PPARGC1A levels are associated with significant upregulation of genes regulating the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), lipogenesis, and antioxidant pathways. These observations indicate a strong correlation between PPARGC1A transcriptional activity and heightened metabolic energy demands in glioblastoma cells. Moreover, these data imply that modulation of PPARGC1A expression may play a pivotal role in promoting proliferation within glioblastoma cell lines [16-18]. Another important fact is that melatonin research has primarily focused on the effects of exogenous melatonin injection as a potential cancer treatment [8]. These findings prompted us to look into the glioblastoma cell line under deprivation conditions to evaluate whether the concentration of melatonin changes in circumstances of cellular metabolic stimulation or not.

2. Materials and Methods

2.1 Cell Culture

The human glioblastoma cell line U87-MG (NCBI Code: C531) was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, USA), supplemented with 10% fetal bovine serum (FBS; GIBCO, USA) and Penicillin-Streptomycin (GIBCO, USA). Cultures were maintained at 37°C in a humidified incubator under a 5% CO₂ atmosphere [19].

2.2 Serum Shock Process

U87-MG cells were seeded at a density of approximately 3×10^5 cells per well in 6-well culture plates. The serum shock protocol involved two sequential steps: treatment for 2 hours with 50% horse serum in the experimental group or 10% fetal bovine serum (FBS) in the control group, followed by exposure to serum-free medium for 8 hours. After the initial 2-hour incubation, cells were washed and subsequently cultured in serum-free DMEM supplemented with penicillin-streptomycin for the remaining 8 hours. Following completion of the serum shock protocol, the supernatants from each well were collected, centrifuged, and stored at -70°C for subsequent melatonin quantification. Additionally, following cell harvesting by trypsinization, cell pellets were stored at -70°C for later measurement of intracellular melatonin and total RNA extraction for gene expression analysis.

2.3 Melatonin Assay

Melatonin levels were quantified in both cell supernatants and cell lysates using the Elabscience Human Melatonin ELISA Kit (Lot No: 1RDJVQ8SSG). After the addition of standards and samples, the microplate was incubated for 45 minutes at 37°C. Following incubation, the wells were aspirated and washed three times with PBS. Subsequently, horseradish peroxidase (HRP) conjugate was added to each well and incubated for 30 minutes at 37°C. In the next step, substrate reagent was added to each well, and the plate was incubated for an additional 15 minutes at 37°C. The reaction was terminated by adding the stop solution to each well. Absorbance was measured immediately at 450 nm using a microplate reader. The concentration of melatonin in the samples was determined by comparing the optical density (OD) values to a standard curve. Results were expressed in picograms per milliliter (Pg/mL). According to the manufacturer's specifications, the assay sensitivity (minimum detectable concentration) was 9.38 Pg/mL, with a detection range of 15.63-1000 Pg/mL.

2.4 Cell Proliferation Assay

U87-MG cells were initially cultured for 24 hours in a cell culture flask to be seeded into 6-well plates. Cells subsequently underwent the serum shock procedure as previously described [20]. After the initial 2-hour incubation, the culture medium was replaced with serum-free medium containing 3 mM BrdU (1 mL per well). Following an 8-hour BrdU labeling period, both serum shock and control groups were harvested, washed with cold PBS, and centrifuged for 3 minutes at 3,000 rpm at 4°C. Cells were then permeabilized and DNA denatured using 2 N HCl and 0.5% Triton X-100 for 30 minutes at room temperature. After three washes with cold PBS, cells were incubated for 90 minutes in the dark with anti-BrdU antibody (Monoclonal FITC Mouse IgG1, κ Isotype Ctrl (ICFC); BioLegend) in 0.5% Tween-20 and 1% BSA. A final series of three PBS washes was performed, and cell proliferation was assessed by flow cytometry using a blue laser (488 nm).

2.5 qRT-PCR

Total RNA of the cells was isolated by RNx Plus extraction kit (SINACLON, Tehran, Iran) and reverse transcribed into cDNA using the cDNA synthesis Kit (YTA, Tehran, Iran) [7]. Transcript levels were determined by real-time qRT-PCR using SYBR Green qPCR master mix 2 $\times$ (100 rxn-Antibody base) (YTA, Tehran, Iran) and performed in a Real-Time PCR Thermal Cycler. The optimal primers used for PCR were as shown in Table 1. Each amplification was performed in triplicate, and expression levels were calculated using the 2 $^{-\Delta\Delta CT}$ method, with GAPDH serving as the normalization control.

Table 1 The specific primers sequences used for gene expression measurement.

Primer Name	Sequences (5'→3')
GAPDH	Forward: 5'-ACAGTCAGCCGCATCTTC-3'
Gene ID: 2597	Reverse: 5'-CTCCGACCTTCACCTTCC-3'
PPARGC1A	Forward: 5'-CCAAAGGATGCGCTCTCGTTCA-3'
Gene ID:10891	Reverse: 5'-CGGTGTCTGTAGTGGCTTGACT-3'

TFAM	Forward: 5'-CCGAGGTGGTTTTTCATCTGT-3'
Gene ID: 7019	Reverse: 5'-GCATCTGGGTTCTGAGCTTT-3'
BMAL-1	Forward: 5'-AGAGGTGCCACCAACCCATAC-3'
Gene ID: 406	Reverse: 5'-CCTCGGTCACATCCTACGACA-3'
DNM1L	Forward: 5'-GATGCCATAGTTGAAGTGGTGAC-3'
Gene ID: 10059	Reverse: 5'-CCACAAGCATCAGCAAAGTCTGG-3'

2.6 Ethics Statement

This study was approved by the Research Ethics committee of the IR.GOUMS.REC.1398.153.

3. Results

3.1 Effect of Serum Shock on U87-MG Cell Line Morphology and Proliferation

The morphology and growth rate of U87-MG cancer cells can be affected by serum shock. Light microscopy was used to observe the morphological changes of the treated cells at first. After 8 hours of serum-free incubation, we observed differences in the shock group in the number and size of the cells compared to the control group (Figure 1). Because the usual serum shock procedure utilized in this work has previously been shown to have a significant impact on cellular metabolic regulation and circadian activation. The observed morphological alteration could be interpreted in this way.

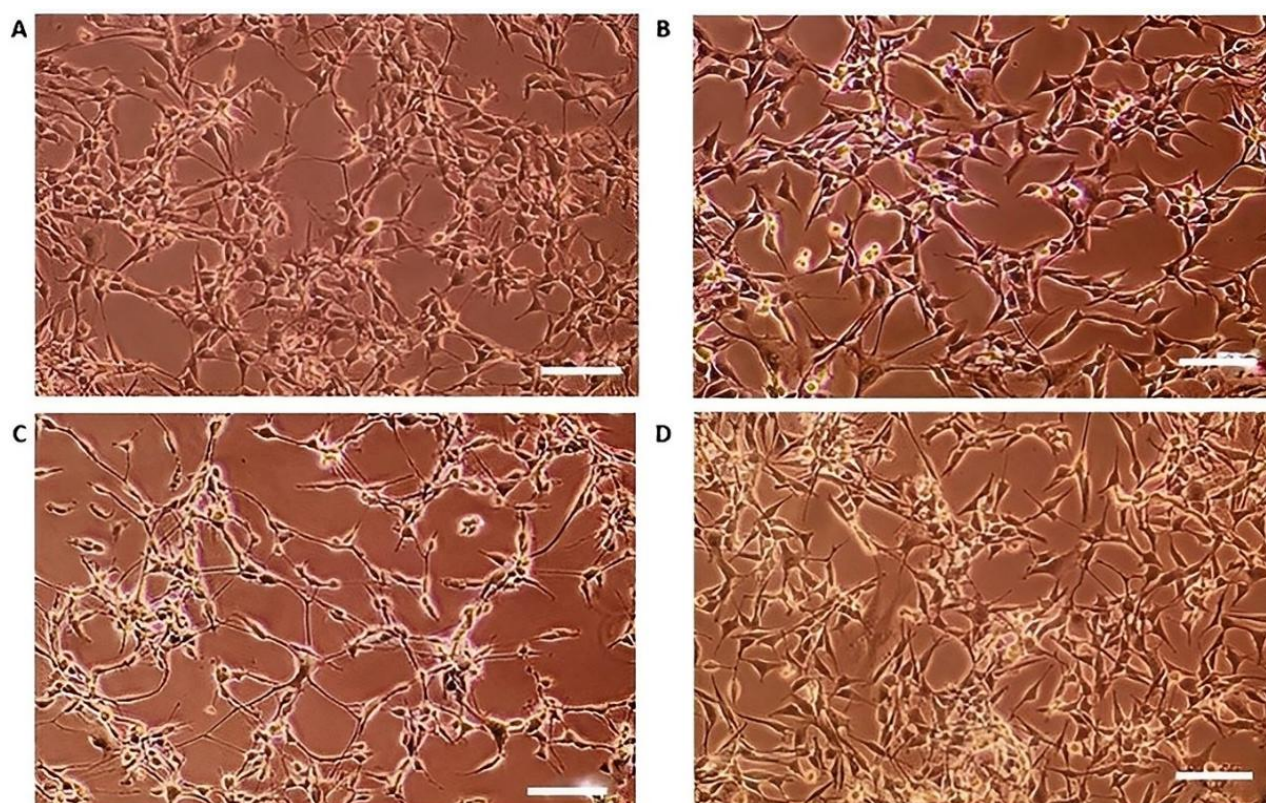


Figure 1 U87-MG glioblastoma cell line morphological changes in response to serum shock. A) Control group after 2 h shock process with 10% FBS, B) Shock group after 2 h shock process with 50% Horse serum, C) Control Group after 8 h in serum-free medium, D) shock Group after 8 h in serum-free medium (Scale bar: 100 μ m).

The proliferative BrdU-positive cells were monitored using flow cytometry to investigate the effect of serum shock on the proliferation rate of the U87-MG glioblastoma cancer cells. Our data revealed that the shock-treated group had a higher proliferation rate, but it was not (Figure 2).

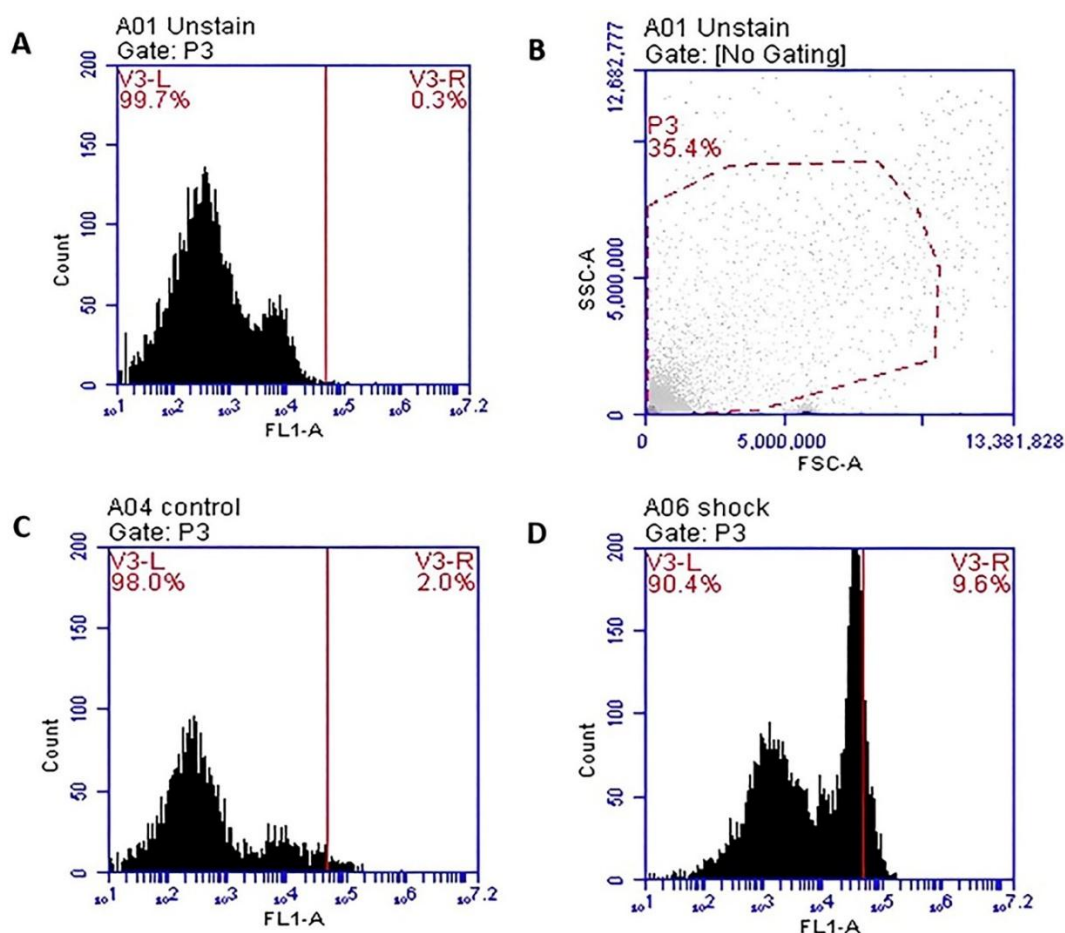


Figure 2 Evaluation of U87-MG cellular proliferation rate by BrdU staining method. The B shows the forward-to-side scatter plots of U87-MG cells. The sample histogram plots are shown for un-stained (B), control (C), and serum shock (D) treated U87-MG cells, in which FL1 indicates BrdU-positive cells. The bar chart shows the mean of BrdU+ and BrdU- U87-MG cells in each group.

3.2 Serum Shock Treatment Increases the Melatonin Concentration in U87-MG Glioblastoma Cells

The intracellular and released melatonin concentrations in the culture medium were determined using the ELISA method to study the effect of serum shock on melatonin production (content) and release in U87-MG glioblastoma cells. With 10+-SE (Pg/ml) in the control vs 35+-SE (Pg/ml) in the shock group ($P < 0.0003$), we found a substantial increase in melatonin concentration in the cell culture supernatant ($P < 0.0003$). (Figure 3A). Furthermore, as shown in Figure 3B, the level of measured melatonin in the cell lysate was larger (~ 10 times) than in the supernatant. The shock group U87-MG cells showed a substantial ($P < 0.0003$) elevated level of melatonin (500+-SE Pg/ml) in contrast to the control (200+-SE Pg/ml).

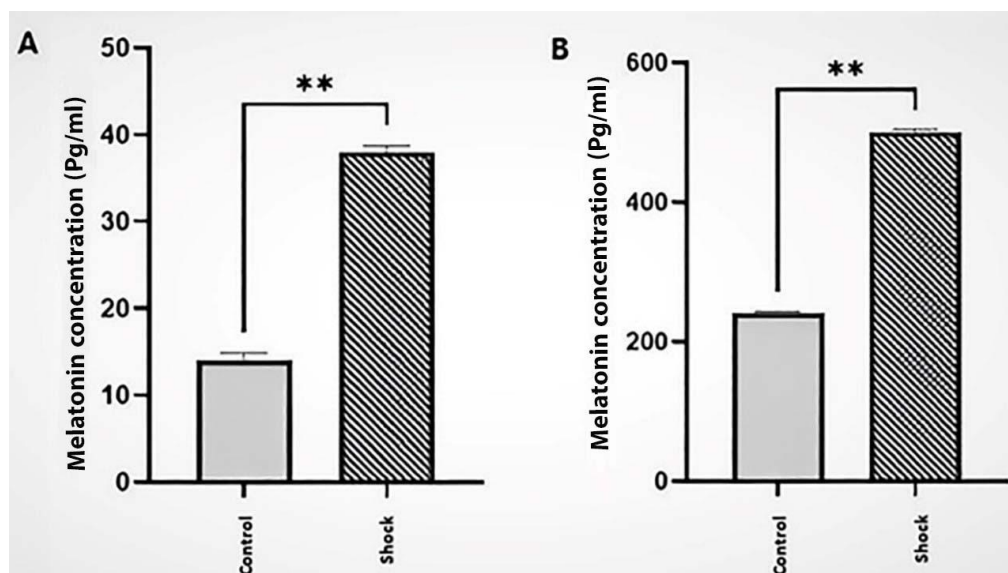


Figure 3 Level of the released (A), and intracellular (B) melatonin concentration in U87-MG cells in response to serum shock.

3.3 Serum Shock Treatment Increases the Mitochondrial Regulator Genes Expression in U87-MG Glioblastoma Cells

The gene expression of BMAL-1, one of the primary circadian regulators, was considerably ($P < 0.0002$) elevated, as expected (2+-SE fold increase) in serum shock-treated cells, showing that the circadian system has been activated (See Figure 4B).

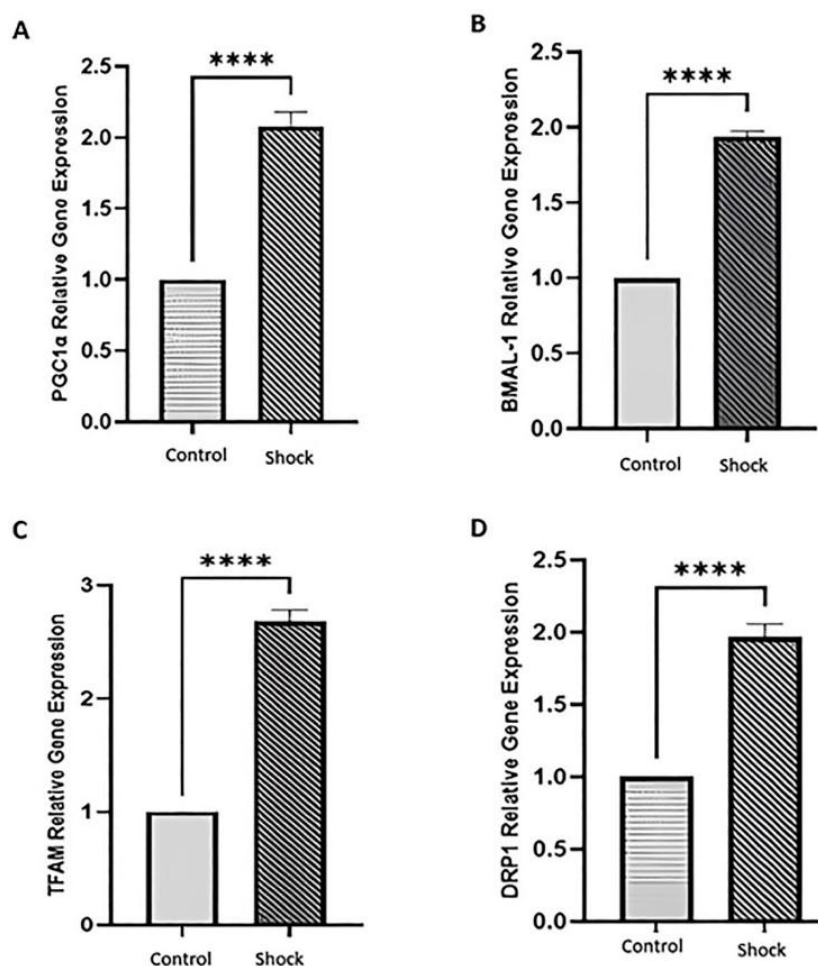


Figure 4 Each amplification was performed in triplicate, and expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with GAPDH as the normalization control. A) The PPARGC1A (PGC1 α) gene expression in the U87-MG shock group and control group. B) The expression of the BMAL1 gene in the U87-MG shock group and the control group. C) The expression of the TFAM gene in the U87-MG shock group and control group. D) The DNM1L (DRP1) gene expression in the U87-MG shock group and control group.

The gene expression experiments showed a significant increase of the mitochondria transcription factor gene, TFAM (2.5 $\pm$ SE fold increase, $P < 0.0002$) in the serum shock group (Figure 4C). Also, the gene expression of the key mitochondrial fission regulator, DNM1L was upregulated in the serum shock-treated U87-MG cells in comparison to the control, (2 $\pm$ SE fold increase, $P < 0.0002$) (Figure 4D) Moreover, the gene expression of the PPARGC1A transcriptional coactivator which is a central inducer of mitochondrial biogenesis was significantly elevated in serum shock U87-MG cells up to 2 folds of gene expression ($P < 0.0002$) (Figure 4A). The fact that serum shock can significantly change the mitochondrial regulator genes, including TFAM, PPARGC1A, and DNM1L, indicates the potential effect of serum shock on the mitochondrial dynamics in response to the metabolic impact of serum shock/starvation treatment.

4. Discussion

One of the established methods for resetting cellular rhythms, which regulate cellular metabolism and circadian rhythms, is serum shock [21]. In our research, the primary variable was the serum shock technique, hypothesized to be a key factor in cellular metabolic reprogramming. As illustrated in Figure 1, cells in the shock group exhibited a higher cell count compared to those in the control group, suggesting that the new conditions promote a more stable environment by modulating the expression of genes associated with mitochondrial function.

Research has demonstrated that astrocyte cells in the rat cortex, as well as glioblastoma cells, can synthesize melatonin independently of pineal cells [22]. Melatonin produced by malignant gliomas exerts an anti-proliferative endocrine effect [23]. We observed that both the melatonin levels in the culture supernatant and the cellular content increased simultaneously in response to serum shock. Notably, the melatonin levels in the shock group were twice as high as those in the control group. This finding may lend support to the hypothesis that U87-MG cells initiate the production and release of melatonin in response to the extreme conditions associated with serum shock.

A recently identified melatonin transporter has emerged as a significant discovery in the field of melatonin research. The PEPT1/2 membrane transporter is responsible for the intracellular transport of melatonin. This protein is located on both the cellular and mitochondrial membranes, which are key sites for melatonin production within the cell. The primary function of this transporter is to regulate melatonin levels between the extracellular and intracellular environments [24]. Our investigation also revealed a significant disparity in melatonin levels between extracellular and intracellular compartments of U87-MG cells, with an approximately tenfold difference. These findings suggest the need for further research to validate the presence of the melatonin transporter in U87-MG cell lines.

The cellular alterations induced by serum shock necessitated examination of specific genes involved in critical cellular pathways. Among these, we analyzed the TFAM gene (mitochondrial transcription factor A), a crucial regulator of mitochondrial gene transcription that also maintains mitochondrial genome integrity and stability. Notably, exogenous melatonin administration in U87-MG cells reduced TFAM expression, leading to mitochondrial genome instability and subsequent apoptosis. This observation underscores the potential role of melatonin in modulating mitochondrial function and cell fate [10]. In our work, when endogenous melatonin concentrations were increased following serum shock, the expression of the TFAM gene increased significantly in the shock group compared to the control group.

The PPARGC1A gene is another one that has been investigated. This gene regulates mitochondrial metabolic activity, which is one of the most essential aspects of cell metabolism. PPARGC1A affects the action of mitochondrial transcription factors and so plays a role. Increased expression of this gene, on the other hand, boosts mitochondrial biogenesis. Previous research has shown that elevated expression of the PPARGC1A gene is associated with the pathogenesis and malignancy of glioblastoma, particularly in the U87-MG cell line [25]. Our results demonstrated significant upregulation of PPARGC1A gene expression, which - when considered alongside the elevated TFAM expression - may provide a mechanistic explanation for the sustained viability of the serum-shocked U87-MG cell population. This coordinated upregulation of both mitochondrial regulatory genes suggests a potential compensatory mechanism that maintains cellular homeostasis under the metabolic stress induced by serum shock conditions. The concurrent

activation of these critical mitochondrial genes may represent an adaptive response that promotes cell survival through enhanced mitochondrial biogenesis and genome maintenance.

The BMAL1-CLOCK protein complex serves as a transcriptional activator for multiple genes, including ROR, Rev-erb, and PPARGC1A. Notably, Rev-erb plays a direct regulatory role in PPARGC1A expression, mitochondrial biogenesis, and autophagy modulation. Furthermore, alterations in the circadian rhythm impact mitochondrial dynamics through changes in DNM1L activity and ATP levels [26]. Based on these established relationships, we investigated BMAL1 gene expression following serum shock. Our analysis revealed significant upregulation of BMAL1 expression, mirroring the increased expression patterns observed for TFAM and PPARGC1A. This coordinated elevation suggests that serum shock and the associated rise in endogenous melatonin levels may simultaneously enhance the expression of multiple genes involved in distinct but interconnected signaling pathways. These findings support the hypothesis that serum shock induces a broad transcriptional response affecting both circadian regulation and mitochondrial function, potentially through melatonin-mediated mechanisms. The parallel upregulation of these key regulatory genes indicates a synchronized cellular adaptation to the metabolic challenges posed by the serum shock condition. Studies have shown that excessive upregulation or suppression of PPARGC1A gene expression disrupts mitochondrial biogenesis and dynamics. However, a moderate increase in PPARGC1A expression can enhance mitochondrial dynamics stability by binding to the DNM1L promoter and upregulating its expression [27]. Following the increase in PPARGC1A gene expression, we decided to evaluate the expression of the DNM1L gene in our investigation. The discovery of a significant increase in DNM1L gene expression demonstrated that the serum shock process leads to the regeneration of cellular metabolism, in which mitochondria play a critical role.

5. Conclusion

Following serum shock, U87-MG glioblastoma cells exhibit marked melatonin synthesis. This endogenous melatonin production coincides with the upregulation of nuclear-encoded mitochondrial genes (e.g., PPARGC1A, TFAM, DNM1L) and circadian regulators (BMAL1), suggesting a coordinated interplay between melatonin signaling, metabolic reprogramming, and circadian synchronization.

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Author Contributions

SB, and MF carried out the cell experiments, gene expression assays and prepared the manuscript. MSJ, NR contributed to bioinformatics analysis of data. SB, MSJ, and NMS contributed to project design. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

All data generated or analyzed during this study are included in this Manuscript.

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