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Analysis and Evaluation of the Effects of Melatonin on Oral Keratinocytes: A Pilot Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Objective: Twenty to 30 percent of children in cross-sectional studies have significant bedtime problems or night waking. Melatonin, a synthetic form of the hormone produced by the pineal gland as a biomarker of the circadian system, is a commonly used nonprescription pharmacologic treatment for sleep disorders in children. Many studies have demonstrated the effect of melatonin supplementation on sleep duration and sleep quality, which can improve overall systemic health and disease prevention. However, despite the growing number of studies demonstrating the effects of melatonin to improve disordered sleep, no available studies have evaluated the effects of melatonin on normal oral tissues. Based upon this lack of knowledge, the primary objective of this study is to evaluate any potential effects of melatonin on normal oral cells and tissues within the physiologically relevant (supplementation) range.

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Methods: Normal oral keratinocytes (OKF4) and human gingival fibroblasts (HGF-1) were obtained and cultured for this study. Melatonin was administered in 96-well growth assays at supplement-equivalent physiologic concentrations at the low, mid and high range (1, 5 and 10 ug/uL) to determine any effects on cellular growth and proliferation. Changes in cellular viability and expression of cell cycle and apoptosis-related pathways were also evaluated.
Results: Curvilinear U-shaped dose responses were observed in OKF cells under melatonin administration, ranging from -11.4% (low), to a maximum of -13.6% (mid) and -5.0% (high) compared with non-treated controls, p=0.029. Dose-responses among HGF-1 cells ranged from +12.1% (low), +17.4% (mid), and +5.0% (high), p=0.021. No changes in cellular viability were observed between control and experimental cells. However, qPCR screening of total RNA revealed significant changes in cell cycle related pathways, including c-myc, GAPDH and P53 but no changes in any apoptosis-related pathways, including Bcl-2, Bax, caspase-3, caspase-8 and caspase-9.
Conclusions: This study demonstrated that melatonin does affect growth but not viability among these cell lines, which was found to be dose-dependent. These results suggest that melatonin may have some limited effects on oral tissues that may influence wound healing and repair but may not affect normal physiologic function or other cellular pathways. In agreement with other pediatric literature supporting the safety of melatonin use, this pilot study does not reveal any deleterious effects that would caution against its use in children or adults.

Keywords: Melatonin; oral keratinocyte; gingival fibroblast; growth.

1. INTRODUCTION

Sleep profoundly impacts virtually every aspect of a child's physical and mental health, daily functioning, and well-being [1]. Thus, it is not surprising that insufficient, disrupted, and poor-quality sleep is one of the most common complaints raised by parents to their pediatric practitioners [2,3]. About 25% of children overall experience some type of sleep problem [4,5]. The majority of these sleep problems in children and adolescents can be managed with behavioral therapy alone; however, there are clinical situations in which pharmacologic intervention (or a combination therapies) is recommended [6-8]. There are a variety of medications used in clinical practice by healthcare practitioners, as well as by parents, to treat pediatric sleep disorders [9,10]. While there is currently no approved sleep medication approved by the Food and Drug Administration for use in children, there is a fair amount of pediatric literature on the safety and efficacy of melatonin as a sleep aid [11,12].

Melatonin is a hormone produced by the pineal gland that has been determined to be a significant modulator of circadian rhythms and the diurnal day-night sleep cycle [13,14]. The circadian rhythm has been demonstrated to function in almost all cells and tissues, although the most widely studied aspects of these functions have focused on the mechanisms and effects in the hypothalamus and associated areas of the brain that regulate sleep, physiology, metabolism and behavior [15,16]. Many studies have demonstrated that dysregulation of melatonin is a critical aspect of sleep and circadian rhythm disorders, which have dramatic and debilitating effects on these patients [17-19].

Many studies have evaluated the positive effects of melatonin supplementation on sleep duration and sleep quality, which can improve overall systemic health and disease prevention [20,21]. Although most sleep dysfunction studies have traditionally focused on these disorders among the adult population, new evidence has suggested that a significant proportion of the pediatric population may also suffer from sleeplessness, insomnia and other sleep-related disorders that may be successfully treated with melatonin supplementation [22-24]. Melatonin may be among the most preferable pharmacological interventions for pediatric patients, as other therapeutic treatments used in adults such as benzodiazepine receptor agonists and sedating antidepressants may have significant and yet unknown effects on developing pediatric brains [25,16].

However, the cellular effects of melatonin are not restricted to the central nervous system and have been demonstrated in various tissues, including both cardiac and reproductive systems [27,28]. Other systems previously thought to be only minimally affected by melatonin, such as the eye, have recently been shown to be significantly

impacted by circadian dysfunction and melatonin dysregulation that greatly increase risk for ocular disease [29,30]. In addition, more complex interactions between the immune system and gastrointestinal tract are now known to be modulated by both endogenous and supplemented melatonin [31,32].

Another area of research focus has been the effects of both endogenous and supplemented melatonin on oral health and tissues, including effects on preventing or treating periodontitis and periodontal inflammation [33,34]. In fact, melatonin levels are known to mediate and modulate diverse oral functions, such as dental pulp stem cell proliferation and salivary production [35,36]. However, despite the growing number of patients being treated with melatonin for oral and other systemic disorders - few studies have evaluated the effects of melatonin on normal oral tissues, such as oral keratinocytes and human gingival fibroblasts [37,38]. Therefore, the primary objective of this study is to evaluate any potential effects of melatonin on normal oral cells and tissues within the physiologically relevant (supplementation) range.

2. METHODS

2.1 Cell Cultures

Cell cultures for this study were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Normal oral keratinocytes (OKF4) and human gingival fibroblasts (HGF-1) were obtained and cultured for this study. In brief, cells were thawed and centrifuged at 2,100 x relative centrifugal force (RCF) to pellet the cells. The supernatant containing dimethyl sulfoxide (DMSO) was removed and cells were resuspended in Dulbecco's Modified Eagles' Medium (DMEM) containing 4.0 mM L-glutamine, 4.5 g/L glucose and 110 mg/L sodium pyruvate, supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin antibiotic solution from ThermoFisher Scientific (Fair Lawn, NJ) as recommended by the manufacturer protocol. Cells were maintained in tissue culture-treated flasks in a humidified Biosafety Level 2 incubator supplemented with 5% CO2 at 37°C.

2.2 Reagents

Melatonin (C13H16N2O2) was obtained from Tocris Biosciences (35-505-0) through Fisher Scientific (Fair Lawn, NJ) CAS 73.31-4 with a verified molecular weight (MW) of 232.283, as previously described [39]. Melatonin was suspended in DMEM cell culture media (described above) using supplement-equivalent physiologic concentration at the low-, mid- and high-range corresponding to 1.0, 5.0 and 10.0 ug/uL, which approximates the range of physiologic and bioavailable concentrations of melatonin found in saliva and serum following over-the-counter supplementation [40-42]. Negative controls were created using media (DMEM) without the addition of melatonin.

2.3 Cellular Viability

Viability of cells was assessed using the Trypan Blue viability assay and a BioRad TC20 cell counter (Hercules, CA). In brief, this assay allows for the standardized and repeated measures of viable or live (unstained) and non-viable (stained) cells with and without experimental treatment, as previously described [43]. Absolute and relative percentages of live cells, as well as cell densities and concentrations were obtained for comparison between control and experimental assays.

2.4 Growth and Proliferation Assays

Melatonin was utilized in 96-well growth assays at concentrations equivalent to those achieved through supplementation-derived serum concentrations at the low-, mid- and high-concentration range (1, 5 and 10 ug/uL) to determine any effects on cellular growth and proliferation. Cells were seeded at standard concentrations of 1 x 10^5 cells/mL and allowed to adhere for at least one hour. Media was removed and either experimental media (with melatonin) or negative control (standard media) was used. Cells were grown for 24 hours (1 day), 48 hours (2 days) or 72 hours (3 days) and were subsequently fixed with formalin, stained with Gentian violet and read using a BioTek ELx808 microplate reader (Winooski, VT) at A630 nm absorbance. Results were exported and analyzed using Microsoft Excel.

2.5 RNA Extraction

RNA was extracted from both control and experimental cells using the ABgene Total RNA isolation kit consisting of phenol: chloroform extraction reagents from ThermoFisher Scientific (Fair Lawn, NJ), as previously described [43, 44]. Briefly, cells were lysed using the phenol: chloroform reagent and centrifuged at 4°C to

separate the RNA-containing aqueous upper phase and the protein-containing lower phase. The upper phase was transferred to a new microcentrifuge tube and RNA precipitated with an equal volume of isopropanol. The precipitate was washed with molecular grade ethanol (EtOH) from ThermoFisher Scientific (Fair Lawn, NJ) and resuspended in nuclease-free water. Purity and concentration was determined using a NanoDrop spectrophotometer at absorbances of A260 nm and A280 nm.

2.6 qPCR Screening

RNA was converted into cDNA for screening and analysis using the ABgene Reverse iT One-Step RT-PCR kit from ThermoFisher Scientific (Fair Lawn, NJ) and a Master cycler gradient thermocycler from Eppendorf (Hamburg, Germany) using a reverse transcription reaction for 30 minutes at 47°C. qPCR screening was accomplished using 20 uL reactions using SYBR green Master Mix from ThermoFisher Scientific (Fair Lawn, NJ). Each reaction was made of 12.5 uL of 2X ABsolute SYBR green master mix, 1.75 uL each of forward and reverse primers, 1.5 uL of sample (diluted to a standard concentration of 1.0 ng/uL) and 7.5 uL of nuclease-free water. Settings for each reaction included enzymatic activation sequence for 15 minutes at 95°C, followed by the standard 40 cycles of denaturation for 15 seconds at 95°C, annealing at the primer pair-specific annealing temperatures [45] for 30 seconds and extension for 30 seconds at 72°C.

Internal qPCR control
Beta actin forward; 5'-GTGGGGTCCTGTGGTGTG-3'; 18 nt, 67% GC, Tm: 69°C
Beta actin reverse, 5'-GAAGGGGACAGGCAGTGA-3';18 nt, 61% GC, Tm: 67°C
Optimal Tm: 62°C
GAPDH control primers
GAPDH forward: 5′ATCTTCCAGGAGCGAGATCC-3′; 20 nt, 55% GC, Tm 66°C
GAPDH reverse: 5′ACCACTGACACGTTGGCAGT-3′; 20 nt, 55% GC, Tm 70°C
Optimal Tm: 61°C
c-myc forward: 5'-TCCAGCTTGTACCTGCAGGATCTGA-3'; 25 nt, 52% GC, Tm 72°C
c-myc reverse: 5'-CCTCCAGCAGAAGGTGATCCAGACT-3'; 25 nt, 56% GC, Tm 72°C
Optimal Tm: 68°C
p53 forward: 5'-ACCAGGGCAGCTACGGTTTC-3'; 20 nt, 60% GC, Tm 70°C
p53 reverse: 5'-CCTGGGCATCCTTGAGTTCC-3'; 20 nt, 60% GC, Tm 68°C
Optimal Tm: 63°C
p53 forward: 5'-ACCAGGGCAGCTACGGTTTC-3'; 20 nt, 60% GC, Tm 70°C
p53 reverse: 5'-CCTGGGCATCCTTGAGTTCC-3'; 20 nt, 60% GC, Tm 68°C
Optimal Tm: 63°C
Bcl-2 forward: 5'-CTGTACGGCCCCAGCATGCG-3'; 20 nt, 70% GC, Tm 75°C
Bcl-2 reverse: 5'-GCTTTGTTTCATGGTACATC-3'; 20 nt, 40% GC, Tm 59°C
Optimal Tm: 54°C
Bax forward: 5'-GGTTTCATCCAGGATCGAGACGG-3'; 23 nt, 57% GC, Tm 70°C
Bax reverse: 5'-ACAAAGATGGTCACGGTCTGCC-3'; 22 nt, 55% GC, Tm 70°C
Optimal Tm: 65°C
Caspase-3 forward: 5'-ACATGGAAGCGAATCAATGGACTC-3'; 24 nt, 46% GC, Tm 67°C
Caspase-3 reverse: 5'-AAGGACTCAAATTCTGTTGCCACC-3'; 24 nt, 46% GC, Tm 68°C
Optimal Tm: 62°C
Caspase-8 forward: 5'-GATATTGGGGAACAACTGGAC-3'; 21 nt, 48% GC, Tm 63°C
Caspase-8 reverse: 5'-CATGTCATCATCCAGTTTGCA-3'; 21 nt, 43% GC, Tm 63°C
Optimal Tm: 58°C
Caspase-9 forward: 5'-GTTTGAGGACCTTCGACCAGCT-3'; 22 nt, 55% GC, Tm 69°C
Caspase-9 reverse: 5'CAACGTACCAGGAGCCACTCTT-3'; 22 nt, 55% GC, Tm 69°C
Optimal Tm: 64°C

2.7 Statistical Analysis

Differences in growth, proliferation and viability were measured by instrumentation, therefore differences between control (untreated) and experimental conditions were determined using two-tailed Student's t-tests in Microsoft Excel (Redmond, WA) for statistical significance, which is appropriate for parametric data analysis.

3. RESULTS

The two normal oral cell lines human gingival fibroblasts (HGF-1) and oral keratinocytes (OKF4) were obtained and placed into culture (Fig. 1). Cell growth and viability were measured, which revealed OKF4 and HGF-1 cell viability upon thawing was approximately 88.1% and 92.1%, respectively. Confirmation of cellular morphology was accomplished by light microscopy for OKF4 oral keratinocytes (Fig.1A) and HGF-1 gingival fibroblasts (Fig. 1B).

Prior to the experimental trials in this study, reliability and stability viability for OKF4 and HGF-1 cell cultures was assessed over time (Table 1). These data demonstrated that both cell lines maintained viability within a narrow range 88 - 92%, which was stable over a number of passages. More specifically, viability of OKF4 cells ranged from 88.1% - 89.1% with an overall average of 88.58% +/- 0.396. HGF-1 cells demonstrated viability ranging between 91.0% - 92.1% with an overall average of 91.5% +/- 0.474.

To evaluate any effects of melatonin on cellular growth, cells were growth with and without the addition of melatonin using supplement-equivalent concentrations across the range that approximates the physiologic and bioavailable concentrations found in saliva and serum following over-the-counter supplementation from the low-, mid-, and high-range corresponding to 1.0, 5.0 and 10.0 ug/uL (Fig. 2). The data demonstrate that melatonin supplementation reduced OKF4 growth in a U-shaped curvilinear dose-response pattern compared with non-treated control cells (Fig. 2A), with some inhibition of growth observed at the low concentration (-11.4%, p=0.027), the highest inhibition of growth observed at the mid concentration (-13.6%, p=0.008), and the lowest inhibition of growth observed at the highest concentration (-5.0%, p=0.036). Comparison of OKF4 growth at each concentration of melatonin compared with non-treated controls was statistically significant, as was the comparison of the controls compared with all experimental melatonin concentrations combined (p=0.029).

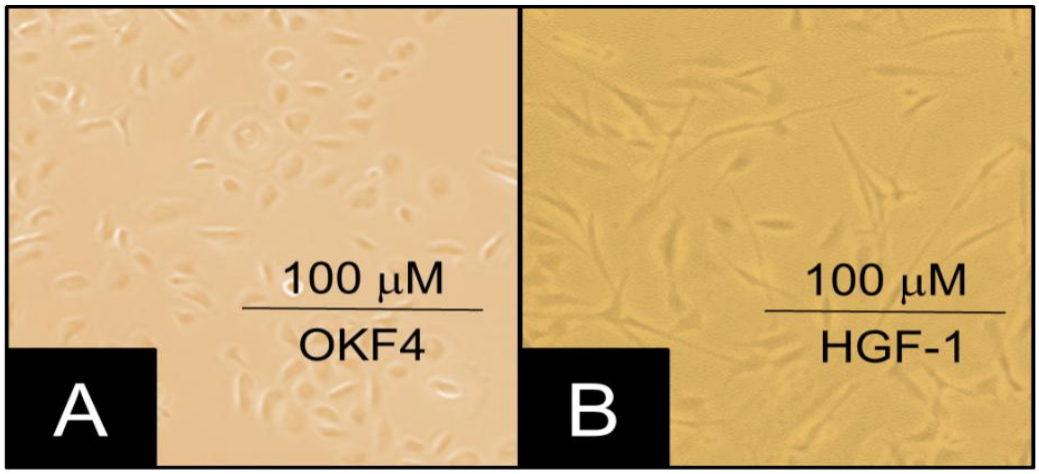

Fig. 1. Establishment of OKF4 and HGF-1 cell cultures. A) Morphology of OKF4 oral keratinocytes (viability 88.1%) and B) Morphology of HGF-1 gingival fibroblasts (viability 92.1%)

Table 1. Viability of OKF4 and HGF-1 cells by passage number

Passage number	OKF4 viability	HGF-1 viability
P4	88.1%	92.1%
P5	88.6%	91.3%
P6	88.3%	91.9%
P7	89.1%	91.0%
P8	88.8%	91.2%
Average	**88.58% +/- 0.396**	**91.5% +/- 0.474**

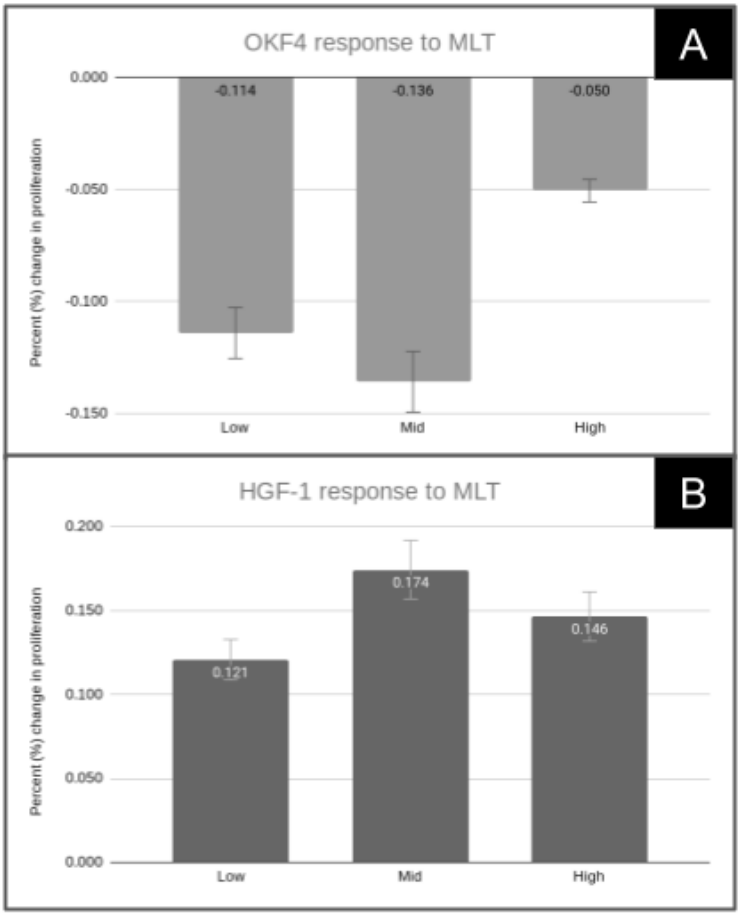

Fig. 2. Cellular growth response to melatonin. A) Curvilinear U-shaped dose-response to melatonin was observed with OKF4 cells, reducing growth by -11.4%, -13.6% and -5.0% over the concentration range tested (p=0.029). B) Curvilinear inverted U-shaped dose-response to melatonin was observed with HGF-1 cells, increasing growth by 12.1%, 17.4%, and 5% at the low-, mid-, and high-concentration levels tested (p=0.021)

However, melatonin administration appears to have a differential effect on HGF-1, inducing an inverted U-shaped curvilinear dose-response pattern compared with non-treated control cells (Fig. 2B), with increased growth at low concentrations (+12.1%, p=0.044), higher growth observed at the mid concentration (+17.4%, p=0.014), and some growth at the highest concentration (+14.6%, p=0.046). Comparison of HGF-1 growth at each concentration of melatonin compared with non-treated controls was statistically significant, as was the comparison of the controls compared with all experimental melatonin concentrations combined (p=0.021).

To evaluate if the effects on growth also exhibited any effect on viability, OKF4 and HGF-1 cell viability was measured in parallel experiments with the growth assays (Table 2). These data demonstrated that although melatonin exhibited growth inhibiting effects on OKF4 cells, no change in viability was observed within the concentration range evaluated. More specifically, viability between the non-treated control cells (average 88.9%) was not significantly different from any of the experimental treated cells at any concentration of melatonin tested (average 89.8%, p=0.10). In addition, although melatonin exhibited growth stimulating effects on HGF-1 cells, no change in viability was observed within the concentration range of these assays. More specifically, viability between the non-treated control cells (average 91.1%) was not significantly different from any of the experimental treated cells (average 91.4%) at any concentration evaluated (average 91.4%, p=0.39).

Table 2. Viability of OKF4 and HGF-1 cells under melatonin administration

Cell line	Viability (CTRL)	Viability (Exp)	Statistical analysis
OKF4	88.5%	[Low MLT] 89.1%	
	89.2%	[Mid MLT] 90.1%	
	88.9%	[High MLT] 90.2%	Two-tailed t-test
	average= 88.87%	**average= 89.8%**	p=0.10
HGF-1	91.2%	92.1%	
	90.9%	91.1%	
	91.1%	91.1%	Two-tailed t-test
	average=91.07%	**average=91.43%**	p=0.39

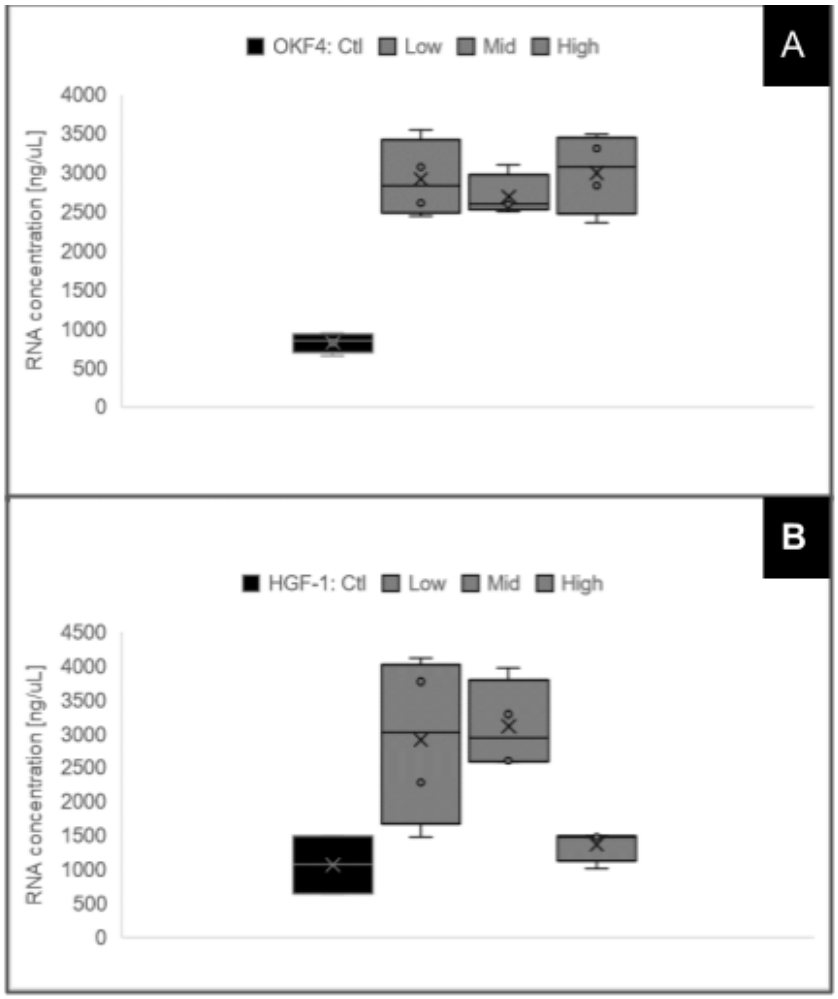

Fig. 3. Total RNA extraction from OKF4 and HGF-1 cells. A) Total RNA extracted from OKF4 control (non-treated) cells increased from 648.9 ng/uL to 2915.4 ng/uL (low), 2571.5 ng/uL (mid), and 2998.6 ng/uL (high), p=0.0001. B) Total RNA extracted from HGF-1 control (non-treated) cells increased from 1069.7 ng/uL to 2908.1 ng/uL (low), 3111.4 ng/uL (mid), and 1473.7 ng/uL (high), p=0.001

Due to the differential effects of melatonin on growth, but the lack of change in viability in either cell line, other mechanisms underlying these observations were explored by analyzing RNA extracted under control and experimental conditions (Fig. 3). These data revealed that total RNA extracted from OKF4 cells was 822.9 ng/uL, which increased significantly under all concentrations of melatonin administration by approximately three-fold (Fig. 3A). More

specifically, melatonin administration increased OKF4 total RNA concentrations to 2915.4 ng/uL (low), 2571.5 ng/uL (mid), and 2998.6 ng/uL (high), p=0.0001.

In addition, total RNA extracted from HGF-1 cells was 1069.7 ng/uL, which also increased significantly under all concentrations of melatonin (Fig. 3B). More specifically, melatonin administration increased HGF-1 total RNA concentrations to 2908.1 ng/uL (low), 3111.4 ng/uL (mid), and 1473.7 ng/uL (high), p=0.001.

The extracted total RNA from each cell line was then used as a template for the creation of cDNA from both control and experimental conditions (Table 3). These data revealed the concentrations of cDNA derived from OKF4 extracted RNA averaged 1012.8 ng/uL, ranging from 864.4 ng/uL to 1096.6 ng/uL. The purity of cDNA measured by the ratio of absorbance at A260 nm and A280 nm averaged 1.82, ranging between 1.81 and 1.84. The concentrations of cDNA generated from HGF-1 extracted RNA averaged 1009.83 ng/uL, ranging from 932.4 ng/uL to 111.2 ng/uL. The purity of cDNA averaged 1.80, ranging from 1.79 to 1.83.

To evaluate any effects of melatonin supplementation on signaling pathways in OKF4 and HGF-1 cells, qPCR screening was performed on the cDNA synthesized from extracted total cellular RNA (Fig. 4). These results demonstrated that all cells (both control and experimental) produced the internal structural control mRNA for beta actin with little variation. However, changes in cell proliferation and growth-related pathways were observed between the control and experimental cells under melatonin administration. For example, the production of the glycolytic pathway enzyme Glyceraldehyde 3-phosphate dehydrogenase or GAPDH decreased significantly among OKF4 cells under melatonin supplementation - corresponding with the decreased rates of growth and proliferation previously observed. In addition, mRNA for c-myc related to the cell cycle progression also decreased in OKF4 cells under all concentrations of melatonin. In contrast, the tumor suppressor and cell cycle regulator protein P53 was increased in OKF4 cells under all concentrations of melatonin supplementation. Finally, no significant changes were observed in the levels of Bcl-2 and Bax, which are key regulators of apoptosis - with no expression of other apoptosis-related pathways, such as caspase-8 (extrinsic pathway), caspase-9 (intrinsic pathway) or caspase-3 (effector).

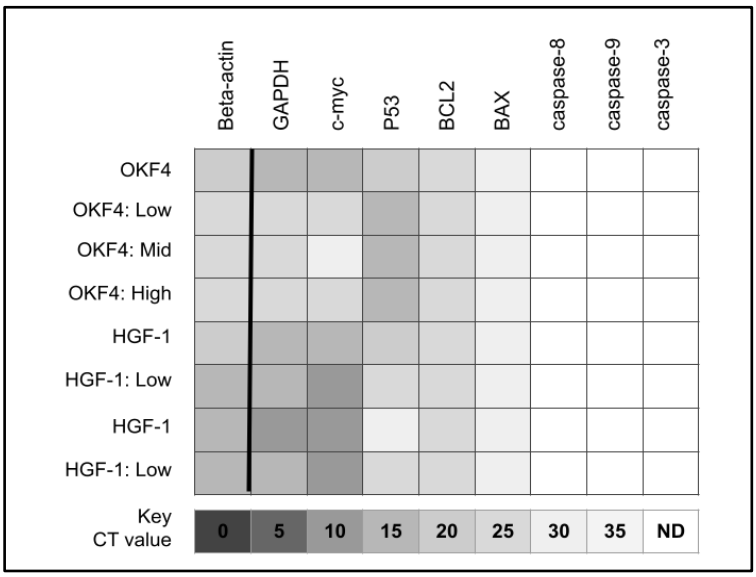

Fig. 4. Heatmap of OKF4 and HGF-1 qPCR screening. Expression of internal structural control (beta actin) was detected in both OKF4 and HGF-1 cells under all conditions. Differential expression of cell cycle regulators GAPDH, c-myc and P53 was observed between control cells and melatonin administration in both cell lines. No differences in BCL2 or BAX expression was observed and no expression of apoptosis-related pathways was observed (caspase-3, caspase-8, caspase-9)

Table 3. Analysis of cDNA derived from extracted total RNA

Cell line	cDNA concentration	A260:A280 ratio
OKF4 - CTRL	974.2 ng/uL	1.84
Low	1077.4 ng/uL	1.81
Mid	1096.6 ng/uL	1.81
High	864.4 ng/uL	1.82
Average	**1012.8 ng/uL**	**1.82**
HGF-1 - CTRL	992.1 ng/uL	1.83
Low	1003.6 ng/uL	1.78
Mid	1111.2 ng/uL	1.79
High	932.4 ng/uL	1.79
Average	**1009.83 ng/uL**	**1.80**

In contrast to the results with OKF4 cells, the production of the GAPDH increased significantly among HGF-1 cells under melatonin supplementation - corresponding with the increased rates of growth and proliferation previously observed. In addition, expression of c-myc also increased in HGF-1 cells under all concentrations of melatonin with decreased expression of the tumor suppressor and cell cycle regulator protein P53. Similar to OKF4 cells, no significant changes were observed in HGF-1 cells and the levels of apoptosis-related pathway regulators Bcl-2 and Bax, caspase-8, caspase-9, or caspase-3.

4. DISCUSSION

The primary objective of this project was to evaluate the effects of melatonin on normal oral cells, including oral keratinocytes and oral gingival fibroblasts, within the concentration range that would be bioavailable following over the counter supplementation. The results of this study demonstrated that melatonin supplementation does appear to modulate growth and proliferation rates of the cells, slowing the growth of oral keratinocytes while increasing the growth of gingival fibroblasts across the same concentration range. This may represent the first such exclusive evaluation of melatonin and its effects on oral keratinocytes and human gingival fibroblasts.

Previous studies on oral keratinocytes have focused mainly on melatonin as an immunomodulator in response to cell injury or insult [46]. For example, melatonin was used to increase survival and reduce inflammatory pathway activation in HaCaT oral keratinocytes in response to ultraviolet (UVB) radiation [47]. In addition, melatonin can decrease inflammatory responses in both oral keratinocytes and gingival fibroblasts in response to photo biomodulation therapy, which has been used to treat oral lesions and other disorders [48]. Finally, stimulation of oral fibroblast production of MMP-9 and TGF-beta by areca nut extract can be modulated, in part, by melatonin administration [49].

Similarly, studies of melatonin among normal gingival fibroblasts have mainly focused on anti-inflammatory properties relating to cell injury or insult [50,51]. For example, studies of commonly used dental adhesives containing 2-hydroxyethyl methacrylate (HEMA) and bisphenol A-Di glycidyl dimethacrylate (Bis-GMA) have induced DNA damage and the associated DNA repair inhibition in these cells, which may be partially restored by sodium ascorbate or melatonin administration [52]. In addition, oral cellular responses to cyclosporine treatment may also be mediated, in part, by melatonin supplementation [53]. Finally, oral cellular responses to Bisphenol-A or BPA, which was widely used in dental sealants and composites, may also be modulated (in part) by melatonin supplementation [54].

Although there is some evidence of the cellular effects and metabolism related to melatonin in HaCaT keratinocytes, much of this relates to ultraviolet light exposure and the mediating role of melatonin in response [55,56]. Most of the rest of the evidence regarding melatonin and the potential cellular effects comes from our understanding of melatonin supplementation and these relationships with circadian rhythms and sleep [57,58]. Of particular interest has been the effects of melatonin on children and adolescents, who may be treated with melatonin for sleep-wake and sleep phase disorders that have recently been observed at increasingly higher rates [59,60]. Research that seeks to understand the effects of melatonin and how normal oral cells and tissues function in response to

melatonin without injury or insult is therefore of increasing importance [61].

5. CONCLUSIONS

In summary, this study demonstrated that melatonin does affect growth (but not viability) among normal oral keratinocytes and gingival fibroblasts, which was found to be dose-dependent with a curvilinear U-shaped response pattern. These results suggest that melatonin may have some limited effects on oral tissues that might influence wound healing and repair but may not affect normal physiologic function or cellular homeostasis, such as induction of apoptosis-related pathways. In agreement with other pediatric literature supporting the safety of melatonin use, this pilot study of these i*n vitro* effects does not reveal any deleterious effects that would caution against its use in children or adults.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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