

*Systematic Review***Additive Benefits of Melatonin on Osteogenic Differentiation Rate and Osteogenic Potential Quantified by Alkaline Phosphatase - A Systematic Review**

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ABSTRACT

Background: Periodontitis is a chronic inflammatory disease affecting the gingiva, Periodontal ligament, cementum and Alveolar bone. Treatment of such a disease is targeted at initial reduction of primary microbial burden followed by repair of existing periodontium and by regenerative procedures wherever required. Bone is a modified form of connective tissue with lifelong dynamic changes in its metabolism. Current advances in the field of regeneration suggest the use of agents which could alter, enhance, speed up or inducing bone formation at desired sites aiding in regenerative therapy and reducing reliance of external substitutes or grafts.

Objective: To establish the additive effect of Melatonin on Osteoblast differentiation using Alkaline phosphatase as an indicator and the time required to record the same in-vitro.

Data Sources: A search was executed in electronic database (i.e., PUBMED CENTRAL, COCHRANE, LILAC, EUROPEAN PMC, SCIENCEDIRECT, GOOGLE SCHOLAR) using following search terms alone and in combination by means of PUBMED search builder from January 1990 up to July 2020.

Study Eligibility Criteria: Studies were stipulate if they met the following criteria: In vivo or In vitro studies comparing the additive effect of melatonin on Osteoblast differentiation rate using Alkaline phosphatase as an indicator were selected.

Study Appraisal and Synthesis Methods: From a total of ten studies four studies claimed additive benefits of Melatonin in increasing Osteoblast differentiation rate or Osteogenic potential amongst Mesenchymal stem cells which was quantified using Alkaline Phosphatase.

Results: The included article suggest without a doubt that Melatonin increases Osteoblast differentiation rate or Osteogenic potential among different Mesenchymal stem cells which was quantified using Alkaline Phosphatase, despite heterogeneity with regards to time duration, concentration and involved cell line.

Conclusion: Although Melatonin was found to have a beneficial effect on osteoblast, myoblast, bone marrow stem cells of different origins, the present systematic review did not furnish concrete evidence to show the exact effect, concentration required, and time taken to visualize these clinical benefits.

Keywords: Bone regeneration; Melatonin; Osteogenesis; Biomarkers; Cellular regeneration

Introduction

Regenerative medicine is currently at its pinnacle with a massive increase in Biomaterials and use of exogenous chemicals to enhance bone growth, however autogenous products which may produce similar results have been overlooked. The long-term but essential delay in testing the safety and approve newer standardized drugs has slowed down the process of drug biosynthesis. Contemporary medicine revolves around the application of pharmacological agents to accelerating the dynamic turnover of bone ranging from the use of vitamins till stem cells. Ectomesenchymal stem cells when introduced at a site promotes the formation of Bone progenitor cells enhancing the formation for bone instead of its destruction [1]. Melatonin, an autogenous hormone secreted from the pineal gland during sleep is one such pharmacological agent that is both biocompatibility and easy to enhance. Melatonin is a local hormone is a nutritional supplement which regulates the sleep wake cycle which is medically prescribed in Rapid eye movement sleep disorders, Parkinson's disease and dementia. In 1999, Melatonin was found to additionally promote Osteoblast differentiation in pre osteoblast cells and rat like osteosarcoma through melatonin transmembrane receptors [2,3]. Ever since newer variants of melatonin have been applied in the treatment of osteopenia, fracture risk reduction in postmenopausal women [4,5].

Rationale of the systematic review

Assessing the level of evidence behind regeneration or proliferation of osteoblasts/ stem cells at a desired sites induced by local application of Melatonin in a controlled environment. These controlled environments include optimum pH, temperature, aerobic nature, humidity along with an osteogenic initiating factor which can only be provided under in-vitro condition.

Objectives

The present systematic review was done comprehensively to include all the articles published where Melatonin was used to induce osteogenic differentiation using Alkaline phosphatase as a biomarker and the time required to record the same in-vitro. Search was carried out using multiple terms including osteogenic potential and osteogenic differentiation rate from 1990 to July 2020.

PICO question

Is there any additive effect of using melatonin on osteoblasts among mesenchymal stem cells in

increasing osteogenic differentiation rate or osteogenic potential using alkaline phosphatase as a biomarker?

P- Osteoblast, progenitor bone cell, bone formation, bone anabolism, osteogenic potential, osteogenesis

C- Melatonin

O- Alkaline phosphatase levels

Methods

Protocol for registration: The present study was registered and reviewed by the ethical committee of institutional Ethical Committee prior to the start of the research with a registration number of IHEC/SDCPERIO/1707/19/077.

Eligibility criteria

Types of studies: Clinical trials, in vitro studies in which Melatonin was used with an objective of increasing osteogenic potential or osteogenic differentiation of osteoblasts.

Types of cell lines: Osteoblasts of human or animal origin, pre-osteoblasts of human or fibroblast origins, fibroblasts of human or animal origin.

Types of outcome measures: Alterations in alkaline phosphatase levels to justify bone turnover, time taken to induce such turnover.

Exclusion criteria: Exclusion criteria for studies were review articles, letters to the editor, chapters from textbooks, abstracts of ongoing research, articles with non-osteogenic functions of melatonin.

Information Sources: For identification of studies included or considered for this review, detailed search strategies were developed for the database searched. The search was carried out from January 1990 to July 2020. All possible databases were searched and for precise selection of studies hand search was also carried out by the assessors involved in the search.

Searched Databases: PubMed advanced search, Science direct, Google scholar, European PMC, Lilac, Cochrane library. Illustrated in Figure 1.

Language: Full text articles in English were only selected.

Hand Search was done from the following journals: Journal of periodontal research, Journal of clinical periodontology, Periodontology 2000, Journal of Indian society of periodontology, Journal of periodontology and restorative dentistry, Clinical oral implant research.

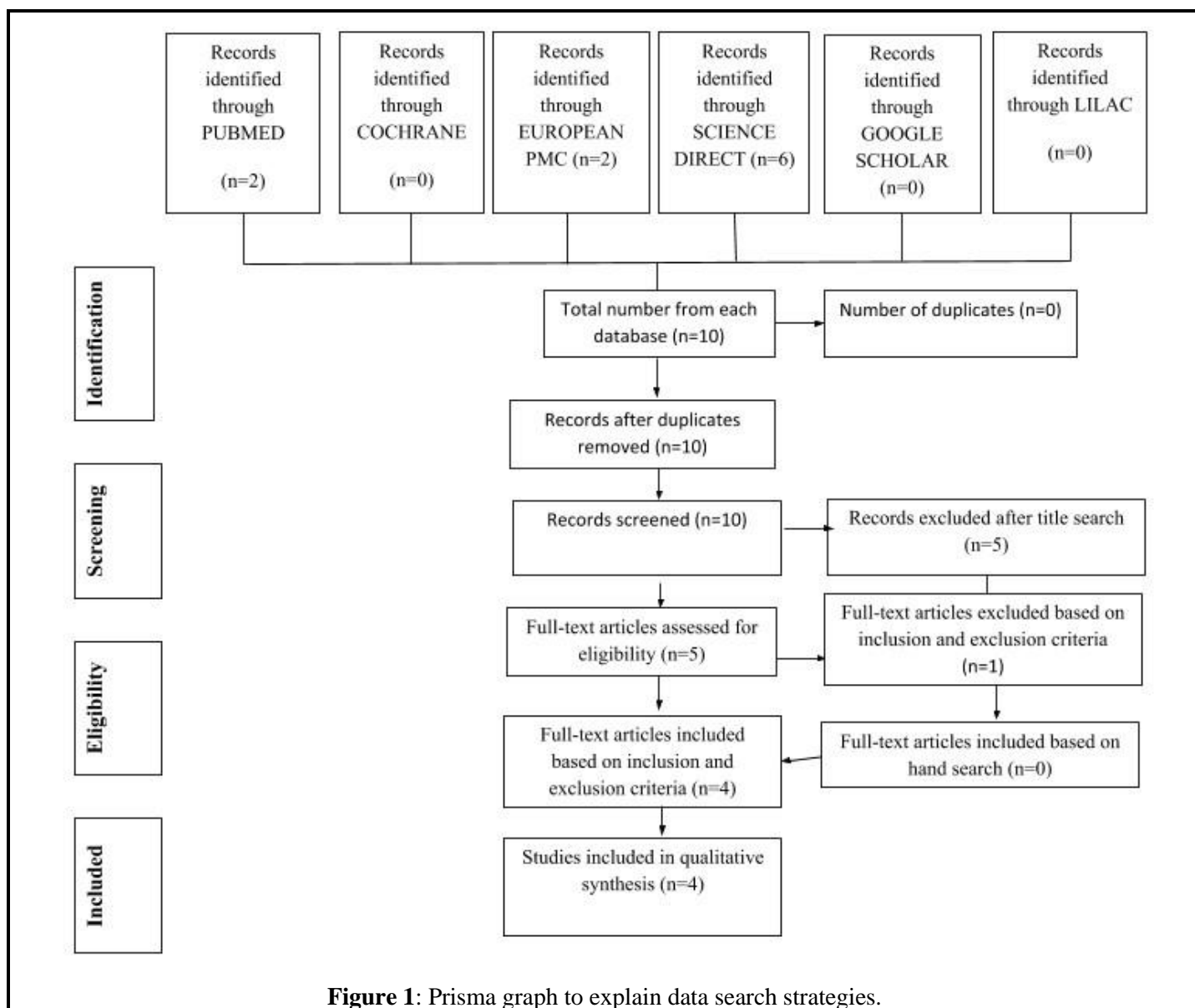


Figure 1: Prisma graph to explain data search strategies.

Table 1: Variables of interest

S. No	Variables of Interest
1	Alkaline phosphatase levels and time taken for clinical observations, concentration of Melatonin.

Table 2: Characteristics of excluded articles

S. No	Author	Year	Reason For Exclusion
1	Yutong-Li et al	2018	Irrelevance based on title
2	Zichuan Ping et al	2015	Irrelevance based on title
3	Maria Letzia T et al	2014	Irrelevance based on title
4	Yoshikazu Mikami et al	2011	Irrelevance based on title
5	Laura Kyllonen et al	2015	Irrelevance based on title
6	Z. Ostrowska et al	1998	Irrelevance based on Abstract And full text reading.

Study selection

The search identified ten publications out of which none were duplicates and five were excluded after title and abstract search. Five full text articles were obtained for studies and evaluated. After evaluation, one was excluded (Table 2) based on inclusion and exclusion criteria. Finally, four were included based on the aforesaid criteria. Following hand search, no other articles were included. Therefore, a total of four publications fulfilled all criteria for inclusion (Figure 1). Variables of interest (Table 1) of the present systematic review were to assess the levels of alkaline phosphatase and time taken for additive clinical benefits of melatonin to be visualized.

Synthesis of results additional analysis

Only quantitative review was possible. Due to heterogeneity of studies included none of the additional analysis were performed. Meta-analysis was not possible due to the heterogeneity of the included studies.

Results

Study selection

The search identified ten publications out of which none were duplicates and five were excluded after title and abstract search. Five full text articles were obtained for studies and evaluated. After evaluation, one was excluded (Table 2) based on inclusion and exclusion criteria. Finally, four were included based on the aforesaid criteria. Following hand search, no additional articles were included. Therefore, a total of four publications fulfilled all criteria for inclusion (Figure 1).

In the present systematic review, all four articles included were in vitro studies using different cell lines of different origins, which compared the additive benefits of different concentrations of melatonin to increase osteogenic potential or osteogenic differentiation rate of osteoblasts using alkaline phosphatase as a marker and the time taken for the same. (Tables 3-5).

Study characteristics & results of individual studies

In twenty-three postmenopausal Osteopenic women, MSDK- a combination of Melatonin, strontium citrate,

Vitamin D3 and Vitamin K2 was administered every night to assess the bone mineral density and quality of life compared to a placebo [6]. MSDK treatment increased bone mineral density in lumbar spine by 4.3%, left femoral neck by 2.2% with increased serum P1NP levels and reduced bone turnover CTx:P1NP with psychometric analysis suggesting improved sleep quality. The same study also exposed human mesenchymal stem cells to MSDK which demonstrated increase in osteoblastogenesis, OPG and decrease in osteoclastogenesis and RANKL levels. In transwell osteoblasts exposed to MSDK there was an increase in pERK1/2, RUNX2 and decrease in ERK5 while no change was seen in the expression of NFkB and B1 Integrin. These findings suggested the use of MSDK for prevention and treatment of Osteopenia, Osteoporosis, and other bone related diseases [6].

A complex containing melatonin along with 2-hydroxypropyl Beta cyclodextrin was used to test Osteogenic differentiation potential of MC3T3-E1 cells which showed increased Alkaline phosphatase activity and mineralized matrix deposition compared to the free MLT treated and untreated cells [4]. The surface characterization of this complex was confirmed by H and C nuclear magnetic resonance spectroscopy and wide-angle X-ray diffraction. These findings suggest the complex action of melatonin with HP-Beta-CD would be a potential formulation for bone regeneration because of its enhanced Osteogenic differentiation efficiency [4].

Mesenchymal stem cells were isolated from bone marrow and fat of adult rats which were cultured in Osteogenic medium in the presence and absence of Melatonin at physiological concentrations of 20-200 pg/ml to check Osteogenic differentiation [7]. After 4 weeks the osteoblast differentiation of Adipose derived stem cells was less than that of bone marrow derived stem cells demonstrated by Alkaline phosphatase activity confirmed by Von Kossa and Alizarin red staining and a higher incidence of apoptosis cells. These findings suggest that bone marrow stem cells have a higher Osteogenic potential compared to adipose stem cells and the use of Melatonin promotes Osteogenic differentiation of Bone marrow stem cells [7].

Table 3: General Information of included articles.

S. No	Year	Author	Source	Group	Parameter	Variable	Methodology and Statistics	Results
1	MAY 2018 Invitro	Masahiko Terauchi et al	Mouse, Osteoblast precursor cells line	-Single Group MC3T3-E1 ^(a) Line -Melatonin 10mg -HP-B-CD ^(b)	Alkaline phosphatase, Alizarin red, GAPDH ^(c)	1)Alkaline Phosphatase-Levels increased with Matrix mineralization 2)Time- checked on 3,6,9 days	-ELISA for Alkaline Phosphatase -ANOVA -Turkey-Krammer test -Fishers	Maximal increase in Alkaline phosphatase was seen on 3rd followed by 6th day and 9th day in both Melatonin alone as well a better result in Melatonin with HP-B-CD.
2	June 2017 Invitro	Younho Han et al	Mouse, Myoblast Cell line	-Single Group C2C12 Lines ^(d) -Mela ^(e) -1MicroM -BMP-4	Osterix, Alkaline Phosphatase.	1)Alkaline Phosphatase-increase similar to the concentration of Melatonin. 2)Time-3,10 days	-ELISA for Alkaline Phosphatase -ANOVA	Minor Increased Alkaline Phosphatase in Melatonin alone group with increase in BMP-4 ^(f) group with additional increase on addition of Melatonin.
3	January 2017 Clinical Trial	Sifat Maria et al	Human, Post- Menopause Women	-Two groups n= 23 Grp 1-MSDK ^(g) (12) 11 Grp 2-Placebo 11	Bone Density, Alkaline Phosphatase, Serum P1NP, Osteocalcin, Osteoprotegrin	1)Alkaline Phosphatase-levels increased with Tissue resistant Acid phosphatase, alizarin Red staining.	-ELISA for Alkaline Phosphatase -Students T-test	Increase in Alkaline phosphatase, tissue resistant Acid phosphatase, ratio of OPG:RANKL

	Invitro			-Melatonin- 5mg. -Str, Vit D ₃ , Vit K ₂	,RANK/RANK L ^(h)	2)Time-21 days	-Welch Correction -Fishers Exact	
4	July 2008 Invitro	Arash Zaminy et al	Adult Rats, MSC ⁽ⁱ⁾	-Single Group MSC Lines -Melatonin 20- 200pg/ml	Osteocalcin, Alkaline Phosphatase, Alizarin red, Von kossa.	1)Alkaline Phosphatase- increased osteoblast differentiation in bone marrow differentiated stem cells 2)Time-4 weeks	-ELISA for Alkaline Phosphatase -ANOVA	Increase in Alkaline phosphatase in Bone marrow stem cells with converse effect on Adipose derived stem cells
<p>(a) MC3T3-E1: mouse osteoblast precursor cell line (b) HP-B-CD: hydroxypropyl Beta cyclodextrin (c) GADPH: glyceraldehyde 3-phosphate dehydrogenase (d) C2C12: mouse myoblast cell line (e) Melatonin (f) BMP 4: bone morphogenic protein 4 (g) MSDK: melatonin-strontium-Vit D3-Vit K2 (h) OPG-RANK: osteoprotegerin-receptor activated nuclear factor Kappa Beta/ligand (i) MSC: mesenchymal stem cells</p>								

Table 4: Variables of interest and summation of results.

S. No	Author and Study Design	Variable of Interest		Methodology to Quantify	Different Concentrations of Melatonin
		Alkaline Phosphatase Concentration	Time*		
1	Masahiko Terauchi et al (May 2018) Invitro	Alkaline Phosphatase- Levels increased with Matrix mineralization	3,6,9 days	ELISA for Alkaline Phosphatase	10 mg ^(a)
2	Younho Han, et al (June 2017) Invitro	Alkaline Phosphatase- increase similar to the concentration of Melatonin.	3,10 days	ELISA for Alkaline Phosphatase	1 MicroM ^(b)
3	Sifat Maria et al (January 2017) Clinical Trial, Invitro	Alkaline Phosphatase-levels increased with Tissue resistant Acid phosphatase, alizarin Red staining.	21 days	ELISA for Alkaline Phosphatase	5 mg
4	Arash Zaminy et al (July 2008) Invitro	Alkaline Phosphatase- increased osteoblast differentiation in bone marrow differentiated stem cells	4 weeks	ELISA for Alkaline Phosphatase	20-200 pg/MicroL ^(c)
<p>*Time period is defined as the point after initiation of the study when test for alkaline phosphatase levels was done, mg: milligrams, MicroM: micro moles, pg/MicroL: picogram per microliter. (a) Mg: milligram (b) Micro moles (c) Pg/MicroL: Picogram per Microliter</p>					

Table 5: Summation of results.

S. No	Title	Statistics	Justification	Significance
1	Sifat Maria et al January 2017	Students T-test Welch Correction Fishers Exact Power-0.80	A priori sample size of 10 was required for significant change in lumbar bone density with 80% power. Comparison of baseline characteristics of MSDK and placebo performed using Students t test for dependent variables and Welch's correction for unequal variance. Pearson's correlation coefficient was carried out for relation between MSDK and bone density, bone markers.	Highest
2	Masahiko Terauchi et al May 2018	ANOVA Turkey-Kramer test Fishers Power-0.95	Statistical difference between the means of individual groups was determined by one-way ANOVA followed by Turkey-Kramer. Statistical significance was present and determined using Fishers least significant difference multiple comparison test	Moderate
3	Younho Han et al June 2017	ANOVA Power-0.95	Data was analyzed using one way or two-way ANOVA, Results expressed as mean value and standard deviation	Moderate
4	Arash Zaminy et al July 2008	ANOVA Power-0.95	Data was analyzed using one-way ANOVA followed by Dunnett's test. All assays performed were triplicate	Moderate

A mouse premyoblast cell line C2C12 was cultured with BMP-4 and transferred with luciferase reporters and melatonin of 0.5 to 1 MicroM concentration different [5]. Alkaline phosphatase was activity of C2C12 was increased significantly in the presence of BMP-4 and further increased by melatonin treatment and in a dose dependent manner, along with dense red staining by Alizarin Red. To assess the effect of Melatonin on BMP-4 induced transcriptional pathway luciferase assay was done which showed an increase in ALP, BSP and OC promoters up regulated at similar concentrations of Melatonin. Such evidence suggests Melatonin directly regulates late-stage osteoblast differentiation by enhancing Osterix expression, and its potential in the treatment of Osteoporosis [5].

The above-mentioned studies confirm the potential of Melatonin at different physiological doses in inducing Osteogenic potential amongst adult rat bone marrow stem cells, mouse pre-myoblast stem cells, human mesenchymal stem cells with sufficient statistical analysis to confirm their findings despite invitro nature of three of the four selected studies. Along with the hypothesized and proven pathway of melatonin stimulated BMP-4 induced transcriptional activity of osteoblast differentiation [8].

Synthesis of results and additional analysis

Only quantitative review was possible. Due to heterogeneity of studies included none of the additional analysis were performed. Meta-analysis was not possible due to the heterogeneity of the included studies.

Discussion

Report on quality of evidence looked upon: Four studies were included in this review all of which had substantially moderate level of evidence excluding one clinical trial along with in-vitro which showed high level of evidence.

Melatonin is a hormone secreted by the pineal gland involved in the sleep-wake cycle, it has different receptors situated all over the body including brain, cardiovascular system, gastrointestinal system, excretory system, cells of the immune system, epithelial cells of the reproductive system with evidence of these receptors providing additional benefits apart from those provided by the pineal gland hormone itself [9,10]. Studies with invitro stem cells have suggested a possible role of melatonin transmembrane receptor's ability to promote osteoblast maturation among preosteoblast in rat like Osteosarcoma [6]. There was also evidence of increased alkaline phosphatase activity among different

human adult Mesenchymal stem cells via MT2 melatonin receptors which was hypothesized as extracellular signal regulated kinase 1/2 pathway [11,12].

In the present systematic review too, most of the studies compared a concentration of melatonin ranging from 20pg/microL to 10mg without any standardized concentration (Table 4). As melatonin is considered as a nutritional supplement that has demonstrated efficacy to renormalize bone marker turnover, testing such agents that increase proliferation of Osteoblasts at a required site or increased differentiation of pluripotent cells which would differentiate into Osteoblasts would require a controlled environment. Once the lacunae of information in regard to the optimal concentration of melatonin, time required to visualize its additive benefits on osteoblasts quantified by bone markers like alkaline phosphatase is fulfilled only can clinical application to assess true periodontal regeneration be initiated. The purpose of this review is to obtain a consensus on optimal concentration of melatonin required for its additive benefits on osteoblasts and the time required to do the same (Table 3).

Consideration was made on inclusion of MSDK in the study by Sifat Maria [7] which utilized a series of chemical agents apart from melatonin based on the justification made by the author that all the individual reagents act independent of one another. Where Strontium citrate is said to increase bone density, reduces fracture tendency in post-menopausal women with Osteopenia and Osteoporosis, Vitamin K2 prevents bone loss, fracture risks by affecting bone microarchitecture and Vitamin D3 increases vertebral bone density [13-15].

Quantitative evidence: Meta-analysis was not possible in the present systematic review, mainly due to the heterogeneity of the studies included. Hence, only quantitative review of studies was possible.

Inference: From this systematic review it can be concluded that Melatonin had an additional benefit of increasing osteogenic potential of osteoblasts, quantified using alkaline phosphatase at different concentrations (20pg/microL to 10mg) based on their origin and nature of cell lines in a time frame ranging from three to twenty-eight days.

Limitations: Studies included assessed only the benefits of using melatonin in a controlled environment,

however periodontal disease is multifactorial in nature with several influencing factors creating a susceptible environment for breakdown of periodontal tissue. Thus, all findings seen in these studies cannot be generalized and blindly accepted, but in combination with adjunctive periodontal therapy may have a potential role in the future. The assessment of bone turnover was done based on alkaline phosphatase alone with no other molecular markers to confirm the same. Although, many other commercial agents have proved to have a beneficial role in bone regeneration, the dearth of studies concentrating on this aspect is low or almost nil. The assessed cell lines and their origins were also heterogeneous to come to a specific conclusive remark.

Future implications: When the present review was considered, most of the research was concentrated on usage in vitro study designs to assess the clinical benefits of melatonin on bone turnover. It would be beneficial if future studies concentrate more on clinical application of these findings in patients with periodontitis to assess its true regenerative potential on bone turnover. As periodontitis is a multifactorial disease, it may not always be possible to achieve consistent beneficial effect using a single therapeutic regenerative agent alone but might also require additional periodontal therapy.

Conclusion

Melatonin was found to have a beneficial effect on osteoblast, myoblast and bone marrow stem cells of different origins, the present systematic review did not furnish concrete evidence to show the exact effect, concentration required, and time taken to visualize these clinical benefits. However future trails have to be done extensively on its application as an ideal periodontal bone regenerative agent.

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Conflict of Interest

The author declares no conflict of interest.

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