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Assessment of preventive effect of melatonin on conversion of oral premalignant lesions to squamous cell carcinoma: An animal study on mice

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ABSTRACT

There is a direct association between survival rate of patients with oral cancer and the stage of the disease at the time of diagnosis. Early preventive actions which stall the progression or reversethe malignant lesions are very important. Different studies have shown the preventive effect of melatonin on progression of skin cancers, fibrosarcoma, endometrial carcinoma and protective effect on hepatocarcinogenesis. The aim of this study was to assess the effect of melatonin in preventing the progression of oral premalignant lesions to malignant lesions and squamous cell carcinoma (SCC). In order to induction of premalignant lesions, all subjects were received 50 mg/ml water-soluble carcinogen 4-nitroquinoline-1-oxide for 14 weeks. Then subjects were divided into 3 groups each containing 8 mice.Group 1 received 2 mg/L, group 2 received 20 mg/L melatonin in drinking water, and group 3 received only water without melatonin as the control group. In week 22, all mice were euthanized; their tongues were totally removed and photographs were taken from all visible lesions. Samples were evaluated to determine dysplasia. Statistical analysis was performed with SPPS statistics version 15 using descriptive analysis (mean, standard deviation, mode, frequency). Kruskal-Wallis test was used to compare the mean differences between study groups, and if the difference was significant, chi-square test was performed. A p value of less than 0.05 was considered statistically significant. Our study findings showed that melatonin couldregress the progression of malignant lesions by reducing its size. Moreover it was found that melatonin is significantly more effective on reducing the size of lesions at lower doses (2 mg/L). Although the dysplastic grade and thenumber of lesions were decreased in melatonin recipient groups, but the difference was not statistically significant. The current study has shown that melatonin dose-dependently reduces the size and diameter of malignant lesions.

Key words: premalignant lesions; squamous cell carcinoma; melatonin

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INTRODUCTION

Oropharyngeal squamous cell carcinoma (SCC) is the sixth highest prevalent cancer in the world (1). Despite the progress ofcancer treatment, the overall survival rate of the patient has not significantly changed during past 20 years. The 5 year survival rate of these patients was 45-50 percent (2).Since the 5 year survival rate has a direct

association with stage ofcancer at the time of diagnosis, thus early diagnostic and preventive actions may cease and in some cases regress the progression of the tumor (3) .The role of the pineal gland on induction of different malignant tumors have been studied in recent years and it was determined that the function of pineal gland is decreased in patients with cancer and the regular circadian rhythm of its main hormone which is melatonin is impaired (4).

Resection of pineal gland may increase the growth of tumoral tissue and consumption of melatonin may reverses this process and inhibits the tumorigenesis effect of carcinogen substances.It is highly probable that melatonin decrease growth of tumoral tissue by inhibiting mitosis and regulating the receptors in the tumoral cells (28). In addition the researches that were conducted to evaluate the effect of melatonin in cancer patients have shown that, in these patients, melatonin was released lesser in comparison with healthy subjects (4, 5).

Greenlee et al., in a review article studied the effect of different anti-oxidant complements on breast cancer treatment, has suggested melatonin as the best choice for treating this cancer (33) .More recent review articles were published by Seely et al (34) in 2011 and Wang et al (35) in 2012. Seely and coworkers (34) investigated the effect of administration of melatonin in addition with chemotherapy, radiotherapy, conservative and palliative therapyon patients' treatment response, toxicity of chemotherapy and 1 year survival rate. Wang and colleagues have evaluated randomized clinical trials (RCTs) which have studied the effect of melatonin on solid tumors on preventionof tumoral tissue growth, 1 year survival rate and the side effects of radiotherapy and chemotherapy. Sánchez-Barcelóand coworkers (36) in 2010,studied uncontrolled trials regarding the effect of melatonin as an additional therapy in cancer treatment. Although all 3 studies reported positive effects of melatonin, but they evaluated only particular tumors which oral cancer was not one of them and due to unknown reasons they omitted some of RCT studies.

There are few studies evaluating oralcancers. Chaiyarit et al studied the effect of DNA damage caused by oral lichen planus as a chronic inflammatory disease, and carcinogenesis of the oral mucosa. They evaluated human tissue samples and found that exogenous induction of a specific type of melatonin receptor called MTNR1A may prevent the occurrence of oral cancer in comparison with samples which had not this type of receptor (37).Also Nakamura and coworkers have pointed the importance of the presence of this receptor with the occurrence of oral cancer and importance of epigenetic studies in this field.

On the other hand, Gómez-Moreno and colleagues (39) in their review article evaluated the effect of melatonin on oral lesions. They have pointed that the effect of radioactive factors on the pathogenesis of premalignant oral lesions like leukoplakia (38), oral lichen planuswas previously investigated by Gómez-Morenoet al. (39), and due to anti-oxidant effect of melatonin, they suggested that the use of melatonin should be considered inthe treatment of oral lesions.

Considering the above findings, this study is sought to investigate the effect of melatonin on preventing the progression of oral premalignant lesions to squamous cell carcinoma (SCC) in an animal study. In the other words, it was trying to find out a suitable way to prevent the occurrence of oral cancer at the diagnosis stage of premalignant lesions.

MATERIALS AND METHODS

Sample Size Estimation

The number of subjects was 8 in each group based on a previous study. (15) Considering the probability of death and subjects loss,15mice were enrolled in each group.

Subjects Characteristics

This study was performed in accordance with NHI protocol and Guide for the Care and Use of Laboratory Animals.Femalemice (Mice c52BLb), which is known as black CS7 were used in the study. (40)

Storage Condition

Subjects were kept at the temperature of 22 ± 2 degrees of Celsius, humidity of 60 ± 5% and light/night cycle of 12/12 hours. Accessible foods were available (40).

Grouping

All subjects received 50 mg/ml water-soluble carcinogen 4-nitroquinoline-1-oxide (4NQO) (4-NQO;Cat# N8141, Sigma, St. Louis, MO) for 14 weeks. Based on previous studies the probability of induced premalignant lesions conversion to SCC is 1:7 which is similar to the conversion probability of non-inducedpremalignant lesions (40) .

The subjects were randomly divided into 3 groups (9). Group 1 received low dose (2 mg/L) and group 2 received high dose(20 mg/L) of melatonin (Sigma, St.Louis; MS) in drinking water. Group 3 received only water without melatonin as the control group (41).

In order to ensure the induction of dysplasia, at the end of 14th week some of the mice were euthanized and autopsies were taken from the mice's tongue. The result of dysplasia evaluation is shown in figure 1. At week 22, all mice were euthanized; their tongues were totally removed and autopsies were obtained. Number and largest size of lesions were recorded and samples were evaluated regarding dysplasia.

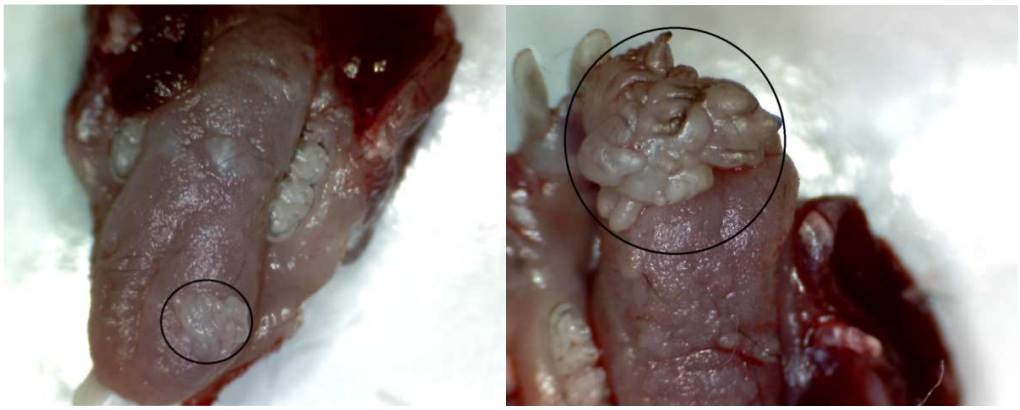

Figure 1. Induction of malignancy. Left figure is belonged to group 1 and right figure is belonged to control group.

Counting the number of lesions and their measurement were performed by project collaborator and fellow student using digital microscopes (Shenzhen Lianshuoda Technology Co., Ltd. China).Samples were fixed in 10% formalin. The prepared blocks were cut out to 50 µm thickness sections and then were stained by hematoxylin and eosin (H&E) stain (figure 2).

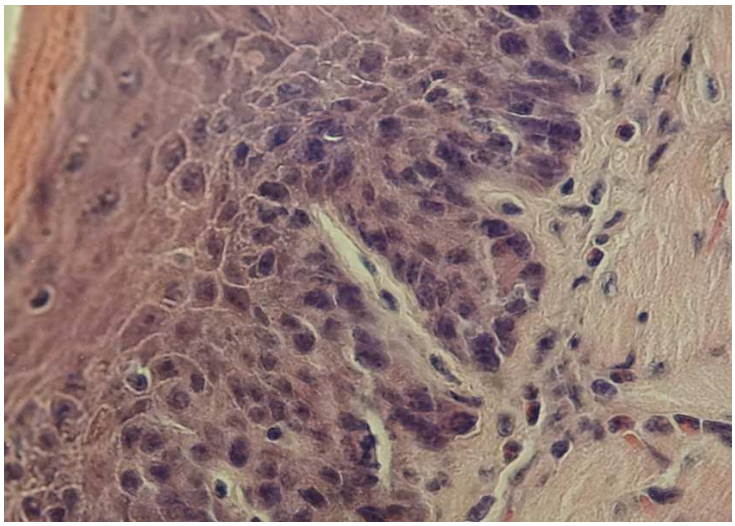

Figure 2.Histopathologic sample of tongue of a control subject

RESULTS

Statistical Analysis

Statistical analysis was performed with SPPS statistics version 15 using descriptive analysis (mean, standard deviation, mode, frequency). Kruskal-Wallis test was used to compare the mean differences between study groups and if the difference was significant, chi-square test was performed. A p value of less than 0.05 was considered statistically significant.

1-Number of lesions

The number of lesions is demonstrated in table 1. To evaluate the statistical significance between the numbers of lesions of each group, the chi-square test was used. It was shown that there was no statistically significant association between the numbers of lesion in the paired groups.

Table1. Number of Lesions in Each group

	Number of Lesions				
	1	2	3	4	5
Control group	2	3	2	0	1
2 mg melatonin recipient	7	0	0	1	0
20 mg melatonin recipient	7	2	0	0	0

2-Diameter of the largest lesion

The results which are demonstrated in table 2 showed that the mean diameter of the largest lesion in the control group was more than other groups and in the 2 mg melatonin recipient group was less than other groups. In order to evaluate the normal distribution of diameter of largest lesion variable, Kolmogorov-Smirnov test was used. The results of this test showed that the diameter of largest lesion variable had normal distribution, thus the data were parametric ($p>0.05$).

Table 2. Mean Diameter of the Largest Lesion

Group	Mean ± Standard Deviation
Control group	2.06 ± 1.05
2 mg melatonin recipient	1.05 ± 0.62
20 mg melatonin recipient	1.07 ± 0.76

The one-way analysis of variance (ANOVA) test was used to evaluate the association between mean diameters of largest lesions of 3groups. The level of significance was defined as p value< 0.05. The results which are demonstrated in table 3 showed that there was a statistically significant association between mean diameters of largest lesions of 3 groups ($p<0.05$). Tukey's test was used to find out which pair groups exactly had the significant difference. The results are shown in table 4.The analysis showed that there was a statistically significant difference between the mean diameter of control group and 2 mg recipient melatonin group, and the mean diameter was higher in the control group than 2 mg recipient group ($p< 0.05$). There was no statistically significant difference between paired groups of control and 20 mg recipient melatonin and also 20 mg recipient melatonin and 2 mg recipient melatonin ($p> 0.05$).

Table 3.Results of One-way Analysis of Variance

Variable	F	P value
Diameter of the Largest Lesion	4.12	0.03

Table 4. Comparison of Lesions' Diameters between Groups Using Tukey'sTest

Group Pairs	Mean Difference	P value
Control with 2 mg melatonin recipient groups	1.01	0.046
Control with 20 mg melatonin recipient groups	0.99	0.053
2 mg melatonin recipient with 20 mg melatonin recipient group	- 0.024	0.99

4-Hispopathology

The results of histopathologic evaluation of the samples are shown in table 5. The findings showed that in the control group the frequency of mice with slight dysplasia was lower than other groups and in the 2 mg melatonin recipient group was higher than other groups. Also the number of subjects with higher dysplasia was more in the control group and was less in the 2 mg recipient melatonin group.

Table 5.Histologic findings according to different group

Group	Number (%)		
	Mild Dysplasia	Moderate Dysplasia	Severe Dysplasia
Control group	0	3 (37.5)	5 (62.5)
2 mg melatonin recipient	4 (50)	3 (37.5)	1 (12.5)
20 mg melatonin recipient	1 (14.3)	4 (57.1)	2 (28.4)

To investigate the significant association between severities of dysplasia of the study groups, chi-square test was used and showed that there is no statistically significant association between severities of dysplasia of the different groups (p> 0.50). Since our data werenormal and parametric, one-way ANOVA test was used to investigatethe statistical significance difference between mean weights. The significance level was considered less than 0.05 .

Catalase (nmoh/min/ml)

The mean and standard deviation are demonstrated in table 6. The results showed that the mean of catalase in the 20 mg melatonin recipient was more than other groups and in the 2 mg melatonin recipient was less than other groups.

Table 6. Mean and standard deviation of each group according to Catalas standard

Group	Mean ± standard deviation
Control group	21.37 ± 3.54
2 mg melatonin recipient	19.25 ± 8.30
20 mg melatonin recipient	23.91 ± 9.39

In order to evaluate the normal distribution of catalase variable, the Kolmogorov-Smirnov test was used. The results of this test showed that catalase variable had normal distribution, thus the data are parametric (p>0.05). Since our data were normal and parametric, one-way ANOVA test was used to investigate the statistical significance difference between means of Catalase. The significance level was considered less than 0.50. The results of the one-way ANOVA analysis showed that there was no statistically significant difference between the means of catalase of 3 study groups (p>0.05).

DISCUSSION

Several studies have shown the effect of melatonin on preventing the occurrence of different cancers.(4) In the current study the effect of melatonin on regression or preventing the progression of premalignant oral lesions were investigated.

In this animal study, the administration of melatonin on drinking water with different doses was performed according to circadian rhythm. This study showed that melatonin could regress the premalignant lesions by reducing their size (diameter of the lesions). Additionally, it was observed that the effect of melatonin on reducing the size of the lesions was dose dependent which was significantly more effective in lower doses (2mg/l). This finding was also observed in Vesnushkin and coworkers study. (41) They evaluated the effect of melatonin with doses similar to our study on benzo(a)pyrene induced skin cancers and found the better effect of melatonin at lower doses. (41) The dose dependent effect of melatonin was also reported in another Vesnushkin and coworkers study (42)about urethane-induced carcinogenesis in the lung. We did not find a study which explains the reason of dose dependent effect of melatonin. It seems that high doses of melatonin side effects such as peroxidation or clastogenic effect may cause this outcome. (43)

Based on our findings, half of our subjects which were treated with lower doses of melatonin had mild dysplasia and more than half of the subjects which were treated with the higher dose of melatonin had moderate dysplasia (58%), but in contrast 62% of the lesions without treatment had severe dysplasia.In other words it seems that the melatonin

receipting subjects shown lower grades of dysplasia but a comparison of their histopathologic findings had no statistically significant differences. According to our study results, in both melatonin recipient groups except 2 mice with 2 visible lesions, other subjects had only 1 observable premalignant lesion, but the difference was not statistically significant. To the best of our knowledge this is the first study which investigating the effect of melatonin on the number and dysplastic grade of lesions. Note that since the previous studies were evaluated the effect of melatonin on cancerous lesions and not precancerous ones, the grade of dysplasia was not evaluated. Melatonin did not regress the malignant lesion to premalignant lesion in any other study. In those mentioned studies, melatonin could prevent the occurrence of cancer on normal tissue or increased the survival of the cancerous animals. (44)

The mechanism of the probable effect of melatonin which prevents or regresses the premalignant lesions is discussed in different ways. Melatoninmay play its anti-proliferative and anti-cancerous role in 5 ways. One of them is the impact on ML1 and ML2 receptors and inhibiting adenylate cyclase which consequently inhibits cAMP and ATP and finally results in decreasing uptake of linoleic acid. (6) On the other way, melatonin affects cellular ML3 receptor and with the help of QR2 enzyme which is a detoxification enzyme, may reduce the chance of occurrence and progression of cancers. (7)Also melatonin may play its preventive role by influencing calmodulin or intranuclear receptors such as RZRB and RZR/RORa and changing the translation of cell proliferation genes. (8) Finally one of the most recognized mechanism of action of melatonin is its anti-oxidant effect.

Melatonin can accumulate active oxygen radicals such as hydroxyl, proxyl, solitary oxygen and nitric oxide radicals, and also can activate anti-oxidant enzymes including superoxide dismutase, glutathione oxidase and catalase which would reduce DNA injury and the occurrence of malignancy. (8, 9) One of the most important effects of melatonin on preventing cancer, which in known from 1993, is its ability to collect free radicals in tissue (melatonin's anti-oxidant effect) that could prevent direct injury to DNA. (45) On the other way it is shown that this hormone would increase the serum level of anti-oxidant enzymes including glutathione peroxidase, glutathione reductase and catalase. (41)One of the most important mechanisms of 4NQO carcinogenesis is due to oxidative stress and then carcinogenesis with free radicals. (46) In the present study, in order to investigate the anti-oxidative effect of melatonin on regression of lesions induced with 4NQO, the serum level of catalase was evaluated in the study groups but the difference was not statistically significant. Evaluation of serum catalase activity in mice under treatment with melatonin was previously performed by Vesnushkin et al. (41)Similar to our study; they found that the serum activity of catalase had no statistically significant difference between high dose recipient melatonin and other study groups. In contrast to our study they found that administration of lower doses of melatonin had decreased activity of catalase.

According to our study findings in comparison with previous studies, in contrast to our expectation, melatonin did not increase anti- oxidative activity of catalase. It is likely that the direct impact of melatonin is responsible for the anti-cancerous mechanism. Additionally, the effect of melatonin on reducing the size and diameter of the lesions may be attributable to its other known mechanism such as anti-mutagenic or anti-tumor effects. It was reported that melatonin could inhibit the mutative effect of some of carcinogen substances like benzopyrene, 7, 12-Dimethylbenz[a]anthracene (DMBA), 2-aminofluorene, 1-2-dimethylhydrazine and bleomycin. On the other way it is possible that the melatonin effect may be due to changing in metabolic activity and liver enzymes. (41, 42) As some studies have demonstrated that melatonin could reduce liver microsomal enzymes activities such as P450 and b5 (47). Anti-angiogenesis effect of melatonin is another specificity of this hormone that may have an impact on reducing lesions' size.

CONCLUSION

The current study has shown that melatonin dose-dependently reduced the size and diameter of malignant lesions but did not statistically significant decrease the grade of dysplasia and the number of lesions.

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