Oncostatic effect of melatonin in head and neck cancer cells: clonogenic assay

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BACKGROUND: THE ONCOSTATIC EFFECT OF MELATONIN HAS BEEN PREVIOUSLY DESCRIBED AMONG DIFFERENT NEOPLASTIC TYPES. ONE OF THESE IS HEAD AND NECK SQUAMOUS CELL CANCER (HNSCC) WITH A HIGH RATE OF MORTALITY AND MORBIDITY. MELATONIN (aMT) COULD CAUSE CELL DEATH IN THIS NEOPLASTIC CELL TYPE. TO DETERMINE THIS, WE PERFORMED A CLONOGENIC ASSAY WITH CAL-27 CELLS TREATED WITH MELATONIN AND/OR RADIATION.

METHODS: CELLS WERE PLATED IN A 6-WELL PLATE, WITH 800 CELLS PER WELL. ASSAYS WERE PERFORMED AT LEAST TWICE AND EACH TIME IN TRIPLICATES. CELLS WERE ALLOWED TO GROW 15 DAYS TO FORM COLONIES OF AT LEAST 50 CELLS EACH ONE. CELLS WERE TREATED WITH MELATONIN (100, 500, 1000, 1500 AND 2000 µM) ALONE OR IN COMBINATION WITH IRRADIATION (8 Gy). TO VISUALIZE COLONIES, CELLS WERE FIXED IN 100 % ETHANOL ON DAYS 12, 13, 14 AND 15 AFTER THEY WERE PLATED AND STAINED WITH CRYSTAL VIOLET SOLUTION. COLONIES WERE SCORED WITH IMAGE J SOFTWARE.

RESULTS: THE RESULTS CLEARLY SHOW THAT MELATONIN INHIBITS COLONY GROWTH OF CAL-27 CELLS IN A DOSE-DEPENDENT MANNER IN THE GROUPS TREATED WITH MELATONIN ALONE 1500 µM OR IN COMBINATION WITH IRRADIATION.

CONCLUSION: THE RESULTS SHOW THE CAPABILITY OF aMT TO PREVENT COLONY GROWTH AND CAUSING CELL DEATH ON CAL-27 CANCER CELLS, ESPECIALLY WHEN COMBINED WITH RADIATION. THIS IS CONSISTENT WITH PREVIOUS STUDIES ON aMT ONCOSTATIC EFFECTS AND SUGGESTS THAT USAGE OF aMT *IN VIVO* SHOULD HAVE FUTURE CLINICAL APPLICATION.

MELATONIN, IRRADIATION, HNSCC, ONCOSTATIC.

Introduction

Melatonin is a natural indoleamine derived from tryptophan. Though formerly considered a hormone, it is now seen as a pleiotropic, multitasking molecule [1-3]. It has several endocrine, autocrine and paracrine effects [1]. It exerts these effects via membrane (MT1, MT2), cytosolic (MT3/QR2) and nuclear (RZR/ROR) receptors [3,5-7]. In addition, some of its effects are not receptor-mediated [8]. It regulates circadian rhythms in many species, including humans, as well as seasonal changes [3,5,6]. It also has a potent antioxidant effect, both directly as a free-radical scavenger and indirectly, by preserving the integrity of several antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase [3,5,6]. It also shows immunomodulating, endocrine-modulating, antiseptic, antitumoral and oncostatic properties in many experimental conditions both *in vitro* [3,7,9,10] and *in vivo* [4,9]. The molecule also shows little to no toxic effects, being an extremely safe molecule [4,11].

The oncostatic effects of melatonin have been described extensively on several publications [4,6,11]. Melatonin is pro-apoptotic, antiangiogenic, anti-proliferative and it inhibits colony formation on several neoplastic types [9]. These include breast cancer, malignant melanoma, prostate cancer, several gastrointestinal and skin cancers and head and neck squamous cell carcinoma (HNSCC) [7,9,11,12]. HNSCC shows a high rate of morbi-mortality, particularly in its oral variant [13]. However, studies of the effect of melatonin in oral HNSCC are still scarce [4,8,14].

The objective of this study was to evaluate the recovery capacity of HNSCC CAL-27 cells after the treatment with melatonin. We also analyzed the interaction of melatonin with irradiation and the effect on the cells capacity to form colonies.

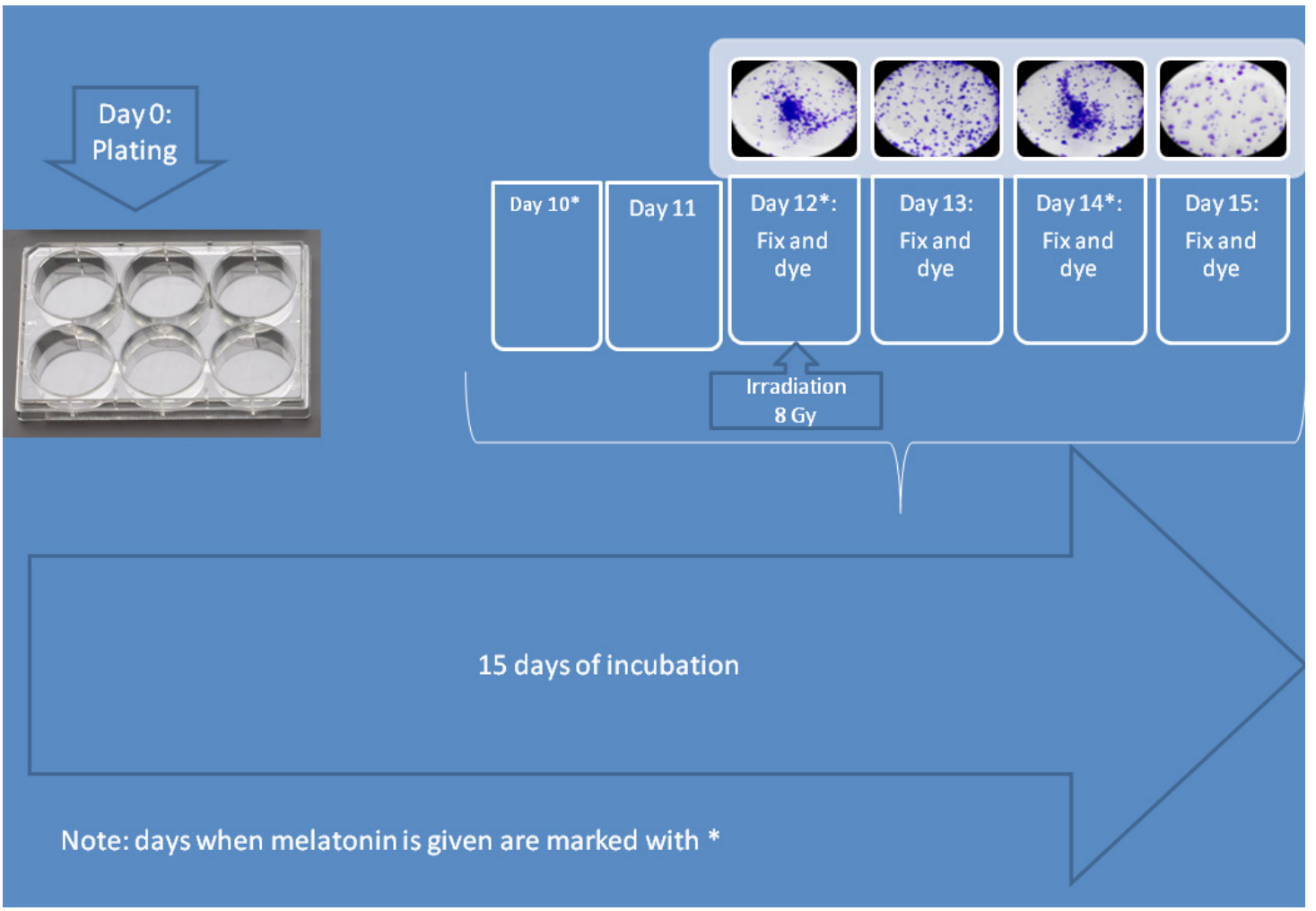
Materials and methods

Cell culture

Cells were plated in a 6-well plate, with 800 cells per well. Then cells were allowed to grow 15 days to form colonies of at least 50 cells each. Cells were maintained in DMEN medium, supplemented with 10% fetal bovine serum at 37º C in a humidified atmosphere of 5% CO_2 and 95% humidity [15,16].

Clonogenic assay

Cells were treated with melatonin (100, 500, 1000, 1500 and 2000 µM) and irradiated with 8 Gy. Three additional cells groups were also established: irradiated only, melatonin-treated only and a control group. The melatonin treatments were repeated every 48 hours. The assay was performed twice and each time in triplicates. Cells were fixed with 100% ethanol on days 12, 13, 14 and 15 after they were treated the first time. They were then stained with crystal violet solution.



▲ **Figure 1**. *The figure shows the chronological development of the experiment, from its beginning, marked as day 0 to its ending on day 15. The days when melatonin was added to the cells are marked with *.*

Image processing

Images of the colonies were taken. The images were processed and the colonies with at least 50 cells were scored using Image J Software.

Statistical analysis

The results of the scoring were analyzed with GraphPad Prism 6 software, using the Bonferroni multiple comparison test at 95% of confidence ($P > 0.05$).

Results

The results clearly show that melatonin inhibits colony growth in a dose-dependent manner in the group of cells treated with melatonin 1500 µM and in the group of cells irradiated and treated with melatonin 500 µM, 1000 µM, 1500 µM and 2000 µM. Colony size was diminished after treatment (Fig. 2). Besides, the group of irradiated cells and the group of irradiated cells treated with low dose-melatonin did not present significant differences in comparison with the control (Fig. 3 and Table 1).

Conclusion

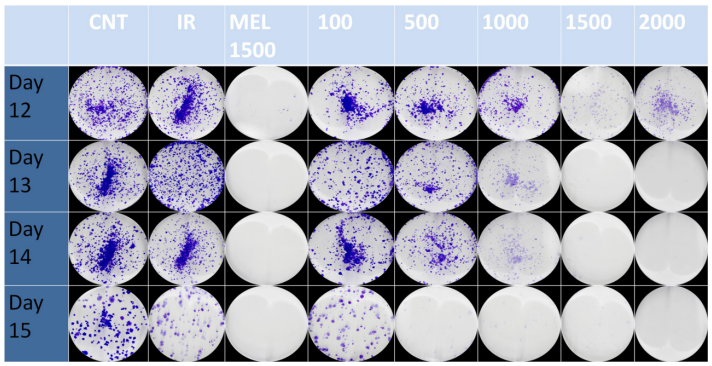
As shown in the results, melatonin inhibits colony growth of CAL-27 cells in a dose-dependent manner in the groups treated with melatonin alone 1500 µM or in combination with irradiation.

The most significant results were found at higher concentrations of melatonin (500, 1000, 1500 and 2000 µM).

Melatonin alone or in combination with irradiation prevents colony formation and proliferation of CAL-27 cells.

Acknowlegements

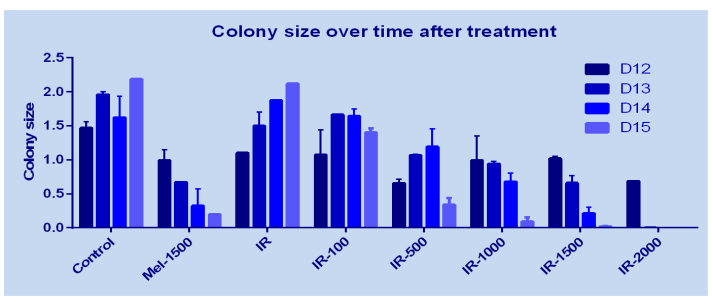
This study was supported by CTS-101 Intercellular Communication Group, Parque Tecnológico de la Salud, Junta de Andalucía, Spain. We would like to thank Sergio García López and Ying Shen Qiang for their collaboration.



▲ **Figure 2**. *The table shows the proliferation and distribution of the CAL-27 cells following the treatments for the control (CNT), irradiated (IR), melatonin 1500 µM only (MEL1500), and 100, 500, 1000, 1500, and 2000 combined with irradiation.*

Day/ Comparison	D12	D13	D14	D15
Control vs.				
Mel-1500	NS	***	***	****
IR	NS	NS	NS	NS
IR-100	NS	NS	NS	NS
IR-500	NS	*	NS	****
IR-1000	NS	**	*	****
IR-1500	NS	***	****	****
IR-2000	NS	****	****	****

▲ **Table 1**. *The table shows the comparison between the means of the number of colonies in each sample against the control over time, from days 12 to 15 after plated (D12-D15). NS for not significant, * for P<0.05, ** for P<0.01, *** for P<0.001, **** for P<0.0001.*



▲ **Figure 3**. *The graphic shows changes on colony size over time, from days 12 to 15 after plating (D12-D15), for each group: Control, irradiated, melatonin 1500 µM only, 100, 500, 1000, and 2000 µM combined with irradiation (IR-100, IR-500, IR-1000, IR-1500, IR-2000).*

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