

The physiological and lifespan alterations in *Caenorhabditis elegans* exposed to different dosages of melatonin

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Abstract: Aging is a process that begins at birth and ends with death. This process is accompanied by environmental effects, which cause structural and functional changes in cells and tissues. With regards to healthy aging, melatonin significantly extends lifespan. This study aims to show the anti-aging effects of melatonin on lifespan by a model organism called *Caenorhabditis elegans*. The nematode strain N2 (wild-type) was acquired, and *E. coli* OP50 was used in the study. Worms were grouped into a control group (n=100), and six experimental groups (group 1, 2, 3, 4, 5 and group 6) (n=100 in each of them). Interventions were made by exposing *Caenorhabditis elegans* to various dosages of melatonin and follow up was made for 21 days. The survey of *Caenorhabditis elegans*, which depends on time and dosage as the main outcome measures, was examined microscopically. Different dosages of melatonin affected the lifespan and morphology of *Caenorhabditis elegans*. Melatonin might be used in the prevention of aging.

Keywords: Melatonin, *Caenorhabditis elegans*, aging.

INTRODUCTION

For a very long time people have been searching for immortality to heal diseases. That is why scientists today search for anti-aging in order to increase lifespan and they will continue this endeavor in the future.

Aging is a process that begins at birth and ends with death. This process is accompanied by environmental effects, which cause some structural and functional changes in cells and tissues. Aging can be classified in two categories as 'primary and secondary'. Primary aging is the structural and functional degeneration that occurs during the aging process, and its speed depends on DNA-preserving mechanisms. DNA-preserving mechanisms protect the cell from free radicals. On the other hand, secondary aging is caused by certain diseases and environmental factors. While delaying the primary aging prolongs the maximal lifespan, reversing the secondary aging extends average lifespan (Cankurtaran 2008).

Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone that is secreted by hypophysis at vertebrates and invertebrates (Hardeland and Poeggeler 2003), and it is secreted out of hypophysis in rats (Sanchez-Hidalgo *et al.*, 2009). Synthesizing of melatonin begins with the hydroxylation of tryptophan-by-tryptophan hydroxylase enzyme to 5-hydroxytryptophan. Then 5-hydroxytryptamine (serotonin) is synthesized (Hardeland and Poeggeler 2003; Sanchez-Hidalgo *et al.*, 2009).

Serotonin, which is acetylated by aromatic 1-amino acid decarboxylase, is then converted into melatonin by hydroxyindol-0-methyl transferase enzyme (Sarlak *et al.*, 2013). Melatonin release is affected by daylight/darkness (Zawilska *et al.*, 2009) and seasonal alterations (Revel *et al.*, 2009). In vertebrates, it plays a role in the regulation of circadian cycles (Stehle *et al.*, 2003; Yamanaka *et al.*, 2014), and orientation to seasonal alterations (Revel *et al.*, 2009; Reiter *et al.*, 2011), and it also functions as an antioxidant agent (Hardeland 2012). Melatonin is provided by placenta in the fetus; its level in the circulatory system alters during lifespan (Waddell *et al.*, 2012) and peaks at puberty (Payne 2006). It is well known that the secretion of melatonin by hypophysis decreases with increasing age (Sharma *et al.*, 1989). In humans and rats, release of melatonin by hypophysis decreases with aging (Hardeland 2010).

In mammals, melatonin acts over G coupled MT1 and MT2 receptors (Hardeland 2009). Expression of melatonin receptors (MT1 and MT2) decreases with aging (Wu *et al.*, 2007). Savaskan and his colleagues reported that age-related neurodegeneration is associated with melatonin (Savaskan *et al.*, 2005). Jung-Hynes *et al.* reported that melatonin might have an influence on re-regulation of degenerated circadian cycle in age-related malignancies (Jung-Hynes *et al.*, 2010).

In this study a model organism N2 (wild type) *Caenorhabditis elegans* (*C. elegans*) species were used. *C. elegans* project was first started by a South African scientist Sydney Brenner in 1965. Brenner and his

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colleagues Robert Horvitz and John Sulston have received 2002 Nobel Prize in medicine and biology for their study on nematode.

There are several studies on *C. elegans* (Sudama 1995; Corsi 2006). *C. elegans* has two basic sex forms: male and hermaphrodite. The hermaphrodite produces sperm and oocyte, and it fertilizes itself. An adult hermaphrodite can release 300 oocytes during lifespan. This nematode is also the first animal whose genomic sequence was determined. *C. elegans* has approximately 20,000 genes and 6 chromosomes, while a considerable amount of these genes are similar to human genes (Olgun *et al.*, 2005).

By studying this model organism, it seems possible to analyze basic problems, such as fertilization, embryonic organization, tissues and organs, neurons and behaviors and aging.

There is evidence that genetic factors influence aging. *C. elegans* is often used in genetic studies related to aging and lifespan. Neurons degenerate and lose the ability of proliferation because of the disorganization of oxidative regulation that occurs with aging (Bishop and Guarente 2007).

In this model study, we aimed at showing the effects of melatonin's anti-aging impacts on lifespan by a model organism called *C. elegans*.

MATERIALS AND METHODS

Study design

Our study design was a randomized, controlled experimental study. We obtained nematode strains N2 (wild-type) of *C. elegans* and *E. coli* OP50 from the *Caenorhabditis* Genetic Center (CGC) at University of Minnesota. We then grouped these worms into one control group (n=100), and experimental groups (group 1, 2, 3, 4, 5 and group 6) (n=100 in each of them).

Preparation of nematode growth media

2,5gr of Peptone, 3gr of NaCl, and 20 gr of Agar were dissolved in 1L distilled water, and autoclaved at 125°C, then cooled to 55 °C. Also 1mL of MgSO₄ (1M), 1mL of Cholesterol (5mg/mL), 1mL of CaCl₂ (1M), and 25mL of KH₂PO₄ buffer (pH: 7) were filtered by a 0,2µm filter, and then added to Nematode Growth Medium (NGM). While the control group did not receive melatonin, the growth media of experimental groups were mixed by a total of 10mL dose of melatonin, and then properly stirred or homogenized. The plates (with a 6cm diameter) were filled by NGM by using sterile procedures, and then cooled. After seeding the NGMs with *E. coli* OP50, they were dried in a sterile cabin (table 1).

Dosages of melatonin

A number of one hundred *C. elegans* were added to 7 individual petri plates after filling the petri plates with NGM. The control group did not contain melatonin, while the experimental groups contained various dosages of melatonin. The Group 1 contained 1000mg/L of melatonin, Group 2 contained 500mg/L of melatonin, Group 3 contained 250mg/L of melatonin, Group 4 contained 100mg/L of melatonin, Group 5 contained 50mg/L of melatonin, and Group 6 contained 25mg/L of melatonin. Homogenization was approved by mixing 10 mL of NGM with each dosage. Even though the control group did not contain melatonin, it did contain NGM (Sutphin and Kaerberlein 2009). In order to prevent fertilization of *C. elegans*, we added fluorodeoxyuridine into NGM. All the experimental studies were held at 22°C.

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS 15.0 (IBM SPSS Statistics 21.0 for Windows, SPSS Inc., Chicago, IL) statistical analysis software. Repeated Measures Analysis of Variance analyzed the differences among groups. The *C. elegans* cumulative survival rate of each group was analyzed by Kaplan Maier Estimator (Kaplan and Meier, 1958). The examination of the relationships within the parametric data of the groups was tested by Pearson's Correlation analysis (table 2).

RESULTS

Lifespan of *C. elegans* exposed to melatonin is shown on table 2, fig. 1 and fig. 2. When compared in terms of vitality, no significant difference was found between the control group and the other groups (Group 1 (1000 mg/L melatonin) and Group 2 (500mg/L melatonin) and Group 3 (250mg/L melatonin)). On the other hand, the survival time (day) and the *C. elegans* count in Group 4 (100mg/L), Group 5 (50mg/L), and Group 6 (25mg/L) were statistically different than the control group (p<0.01, table 2). Besides this, no significant difference was found between Group 4, Group 5, and Group 6 (p>0.05).

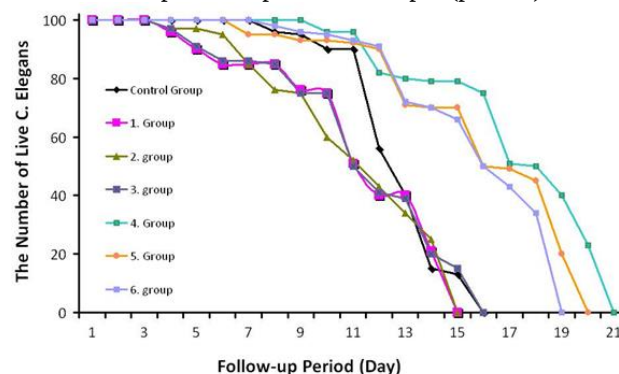


Fig. 1: An illustration of all groups, survival number of *C. elegans* and follow-up period (day). A significant increase

in the survival number of *C. elegans* in plates containing 100mg / L, 50mg / L and 25mg / L melatonin compared to control plate and plates containing 1000mg / L, 500mg / L and 250mg / L melatonin observed ($P < 0.05$).

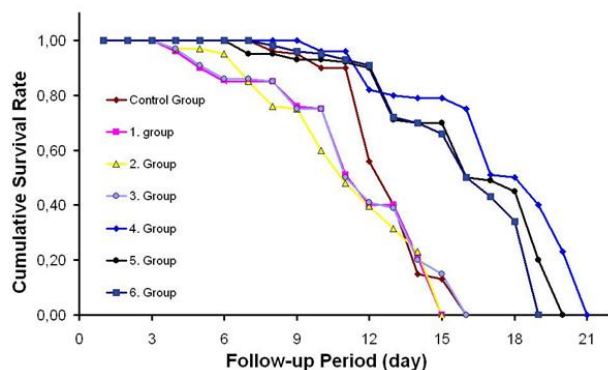


Fig. 2: An illustration of all groups, cumulative survival rate estimated by Kaplan Maier Estimator versus follow-up period. A significant increase in the survival time of *C. elegans* in plates containing 100mg/L, 50mg/L and 25mg/L melatonin compared to control plate and plates containing 1000mg/L, 500mg/L and 250mg/L melatonin observed ($P < 0.05$) fig.

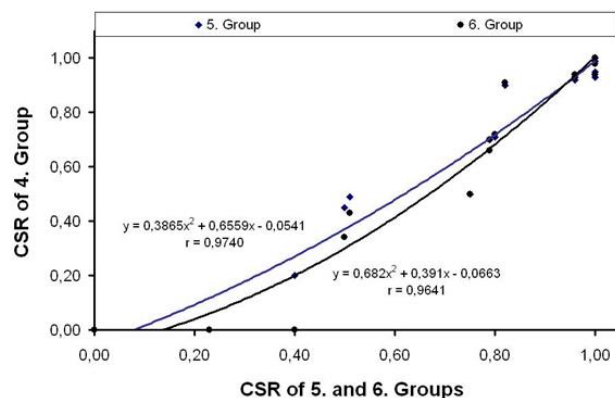


Fig. 3: Pearson correlation graph between cumulative survival rates of 4 group, 5 and 6 groups.

As shown in fig. 2 where the cumulative survival rates were examined, the total count of alive *C. elegans* has dropped to '0' on day 15 in Group 1 (100mg/L melatonin) and Group 2 (500mg/L melatonin), and on day 16 in Group 3 (250mg/L melatonin) and the control group. The *C. elegans* count dropped to '0' on day 21 in Group 4 (100mg/L), on day 20 in Group 5 (50mg/L), and on day 19 (25mg/L) in Group 6. As a result of these findings, we can suggest that the optimal dose of melatonin can be 100 mg/L.

As we analyzed the groups by cumulative survival ratio via the Pearson's Correlation test, we have found a strong relationship between Group 4 and Group 5 ($r: 0.9740$, $p < 0.0001$) (fig. 3). Group 4 and Group 6 had a similar relationship according to the Pearson's Correlation test ($r: 0.9641$, $p < 0.0001$).

We found that the dosages of melatonin such as 100 mg/L and 50 mg/L contributed to the lifespan in *C. elegans*. In addition to this evidence, we also marked less physical damages and better vital functions such as nutrition and movement on *C. elegans* that were exposed to 100 mg/L melatonin (fig. 4).

DISCUSSION

Anatomical and functional degeneration during aging process is occasionally caused by reduced antioxidant capacity and generated free radicals. One of most powerful antioxidant agent is melatonin, and its synthesis decreases with increasing age (Reiter *et al.*, 2001; Cornelissen *et al.*, 2000; Kerman *et al.*, 2005). It is thought that this antioxidant property of melatonin is related to neutralization of oxygen-derived free radicals and acting as protective agent by activation of antioxidant enzymes and inhibition of pro-oxidative enzymes (Reiter *et al.*, 2001; Reiter *et al.*, 1997; Sahna *et al.*, 2006). It has been observed that melatonin causes programmed cell death 'apoptosis' by enhancing antioxidant capacity of the body, and thus can prevent malignancy (Garcia-Santos *et al.*, 2006; Sun *et al.*, 2002; Yoo *et al.*, 2002; Eck-Enriquez *et al.*, 2000). According to recent studies, it has been observed that melatonin acts as a modulator for apoptosis; based upon biological systems it can be both stimulator and both as inhibitor on apoptosis (Toubi and Shoenfeld 2007; Eck-Enriquez *et al.*, 2000).

In our study, the intensifier impact of optimal dosages of melatonin on *C. elegans*' cumulative survival rates can be related to antioxidant capacity and pro-apoptotic effects of melatonin (Toubi and Shoenfeld 2007; Garcia-Santos *et al.*, 2006; Sun *et al.*, 2002; Yoo *et al.*, 2002; Eck-Enriquez *et al.*, 2000).

Significantly increased survival time and motility in Group 4 (100 mg/L melatonin) can be associated with the antioxidant capacity that prevents cell damaging. Also it can be suggested that 100mg/L dosage of melatonin contributes to thermoregulation on biological systems. In other words, we believe that 100mg/L dosage of melatonin leads to loss of heat, and provides metabolic activities in optimal conditions. The study by Macchi and Bruce, which shows melatonin's effects on loss of central body temperature and increased peripheral temperature, corroborates our findings (Macchi and Bruce 2004). In another study, it is shown that low dosages of melatonin injection in rats lead to hyperthermia; and high dosages lead to hypothermia. Also it has been observed that the neurons of thermoregulatory center known as 'preoptic area' (located on anterior portion of hypothalamus) has melatonin receptors, and melatonin leads to loss of temperature by affecting this area (Tsuzuki *et al.*, 2004; Yonel *et al.*, 1996; Nakahara *et al.*, 2003; Cagnacci 1996). All these data suggest that melatonin has both intensifier effects on survival time, and thermoregulation of the body.

Table 1: Study design table of control group and experimental groups and melatonin dosages

	NMG	1000mg/L	500mg/L	250mg/L	100mg/L	50mg/L	25mg/L	<i>C. elegans</i>
C. Group	+							+
1. Group	+	+						+
2. Group	+		+					+
3. Group	+			+				+
4. Group	+				+			+
5. Group	+					+		+
6. Group	+						+	+

C: Control, NMG: Nematot Growth Media

All petri dishes contain fluorodeoxyuridine and *Escherichia coli* OP50.

Each group is composed by 5 petri dishes and individual dish houses n=20 *C. elegans*

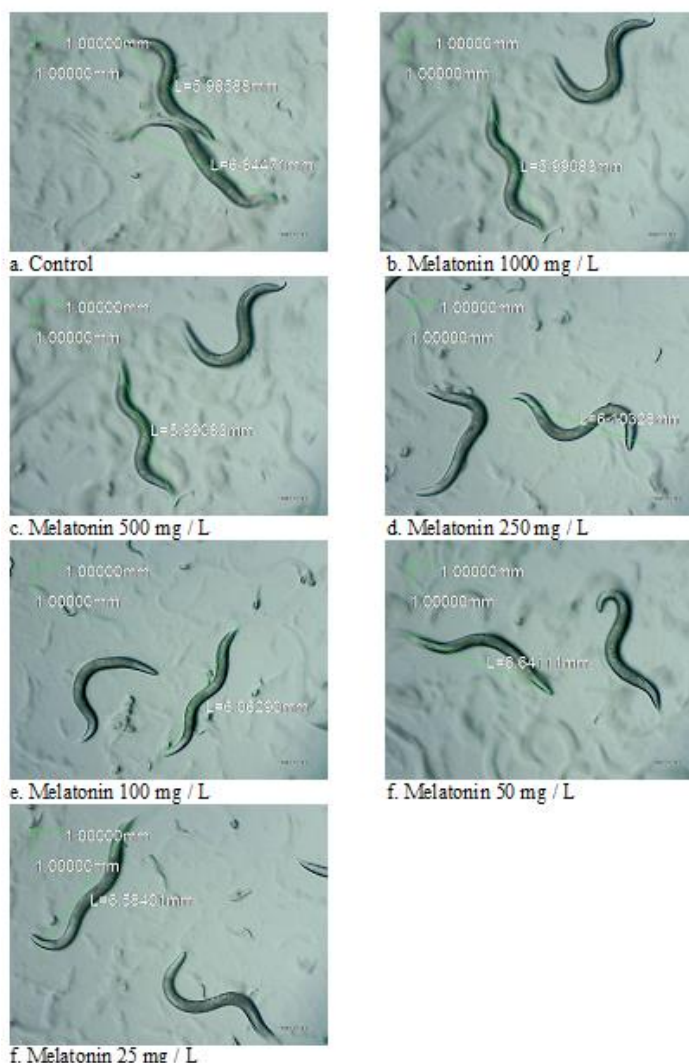


Fig. 4: Micrographs of *C. elegans* from control group and experimental groups that were exposed to different dosages of melatonin.

There are plenty of studies about melatonin's effect on survival. In another study, the effects of melatonin on *Drosophila melanogaster*'s survival time were examined. It was found that 100- μ g/ml dosage of melatonin extended lifespan from 61.2 days to 81.5 days in the control group, because of the antioxidant and free radicals

sweeping effect of melatonin. In our study, the same dosage of melatonin had a positive effect on *C. elegans* survival time (Bonilla *et al.*, 2002).

As a result, our discovery in *C. elegans* provided us significant knowledge about melatonin's posology,

Table 2: Comparison of the data of all groups and the number of live *C. elegans* according to follow-up period (day)

	Control Group	1. Group	2. Group	3. Group	4. Group	5. Group	6. Group
N	100	100	100	100	100	100	100
Follow-up period (day)	21	21	21	21	21	21	21
Mean	56.905	49.714	49.476	50.476	78.619	73.190	71.667
S.D.	45.590	41.673	41.831	41.097	29.234	33.437	36.258
Minimum	0	0	0	0	0	0	0
Maximum	100	100	100	100	100	100	100
MSE	9.949	9.094	9.128	8.968	6.379	7.297	7.912
95% CI from-to	36.152-77.658	30.745-68.684	30.434-68.518	31.769-69.184	65.311-91.927	57.970-88.411	55.162-88.171
P value	* < 0.0001						
Control	-	P>0.05	P>0.05	P>0.05	P<0.001	P<0.01	P<0.01
1. Group	P>0.05	-	P>0.05	P>0.05	P<0.001	P<0.001	P<0.001
2. Group	P>0.05	P>0.05	-	P>0.05	P<0.001	P<0.001	P<0.001
3. Group	P>0.05	P>0.05	P>0.05	-	P<0.001	P<0.001	P<0.001
4. Group	P<0.001	P<0.001	P<0.001	P<0.001	-	P>0.05	P>0.05
5. Group	P<0.01	P<0.001	P<0.001	P<0.001	P>0.05	-	P>0.05
6. Group	P<0.01	P<0.001	P<0.001	P<0.001	P>0.05	P>0.05	-

N: Number of Point, SD: Standard Deviation, SEM: Standard error of mean, CI: Confidence Interval

* P Value for Repeated Measures ANOVA (Analysis of Variance) with post test,

If P value obtained by ANOVA is <0.05, Tukey-Kramer Multiple Comparisons Test (Post-hoc tests) was used to compared all groups.

tolerance and safety. Our findings revealed that optimal dosages of melatonin had favorable impacts on lifespan. High dosages of melatonin had no negative effect on lifespan. The results of this experimental model of the most well known part of the circadian rhythm, melatonin, will help to understand the relationship between antioxidants and the aging process.

CONCLUSION

Our findings suggest that melatonin has anti-aging effects on *C. elegans*, and has potential as a target for anti-aging research.

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