Effects of melatonin and ethanol on the heart rate of *Daphnia magna*

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Melatonin, an endogenous hormone that may regulate circadian rhythms by modulating cholinergic activity, is increasing in popular use as a natural treatment for sleep disorders. However, the effects of melatonin on the human heart are not well characterized, and the consequences of combining alcohol with melatonin are unknown. The myogenic heart of the water flea *Daphnia magna* (*D. magna*) is regulated by inhibitory cholinergic neurons that modulate cardiac function, including heart rate. *D. magna* is a useful model organism for cardiovascular function, due to its physical transparency and susceptibility to cardioactive drugs known to affect the human heart. In this study, the effects of immersion in 10 mg/L melatonin and 5% ethanol on the heart rate of *D. magna* were quantified. Two-hour exposure to melatonin caused a significant decrease in heart rate, from 228 ± 2 bpm to 167 ± 8 bpm. Six-minute immersion in ethanol also significantly depressed the heart rate to 176 ± 10 bpm. Pretreatment with melatonin prior to the addition of ethanol resulted in a greater decrease in heart rate (89 ± 7 bpm) than ethanol or melatonin alone. These findings indicate that melatonin and alcohol may combine to cause a greater depressive effect on cardiac function.

Abbreviations: ACh (acetylcholine), AChR (acetylcholine receptor), bpm (beats per minute), *D. magna* (*Daphnia magna*)

Keywords: cardiovascular function, alcohol, acetylcholine, natural sleep aids, cholinergic regulation

Introduction

Melatonin is a neuroendocrine hormone secreted by the pineal gland. In humans, its primary role is that of a circadian pacemaker, and exogenous melatonin has a well-documented soporific effect when taken during the daytime or early evening (Hughes and Badia, 1997; Pires et al., 2001; Zhdanova et al., 1995). Oral administration of melatonin has been successfully used to treat various sleep-cycle disturbances in children with chronic sleep disorders by decreasing sleep onset latency (Jan and O’Donnell, 1996; Smits et al., 2003). Melatonin may exert these effects by modulating levels of the neurotransmitter acetylcholine (ACh), or by altering the function of acetylcholine receptors (AChR) (Paredes et al., 1999; Brusco et al., 1998; Markus et al., 1996; Zago and Markus, 1999). The heart of the water flea, *D. magna*, is regulated by cholinergic neurons, and may be useful as a model for the effect of melatonin on cardiovascular function (Stein et al., 1966).

*D. magna* are freshwater cladocerans that exhibit a short life span, rapid maturation, and reproduction (Teschner, 1995; Anderson and Jenkins, 1942). Their physical transparency allows for simple observation of internal structures, including the heart (Teschner, 1995; Pirow et al., 2004). In addition, they are highly
sensitive to environmental changes, which cause them to adapt morphologically, physiologically, and behaviorally (Gard et al., 2009; Ikenaka et al., 2006). Water fleas are unusual among crustaceans in that they possess myogenic hearts (Yamagishi et al., 2000; McMahon, 2001). Unlike neurogenic hearts, in which the cardiac ganglion acts as the pacemaker to initiate contraction, myogenic heart rhythms are initiated in the cardiac muscular tissue and are independent of neural, metabolic, or hormonal stimulation (Cooke, 2002; Davis and Hill, 1999). Inhibition by extra-cardiac cholinergic nerves allows for neuronal modulation in addition to the myogenic activity (Stein et al., 1966; Bekker and Krijgsman, 1951; Baylor, 1942). The heart of *D. magna* may therefore resemble the vertebrate myogenic heart more closely than higher orders of Crustacea (Baylor, 1942).

*D. magna* possess a globular myogenic heart inhibited following administration of ACh, which may act on conduction mechanisms in the cardiovascularature (Spicer, 2001; Ellenbogen and Obreshkove, 1949; Prosser, 1940; Stein et al., 1966). Most of the heart is only a single cell-layer thick, which may explain why it is easily influenced by the application of drugs and other substances (Stein et al., 1966). *D. magna* have been shown to respond to a number of cardioactive drugs that are known to affect human heart function (Villegas-Navarro et al., 2003; Postmes et al., 1987). Testing the effects of these drugs is simplified in *D. magna* as the fleas are responsive to pharmacological agents added to the water in which they swim (Campbell et al., 2004). The introduction of these pharmacological agents to water fleas may induce activity directly on the cardiac muscle (Bekker and Krijgsman, 1951).

The ingestion of ethanol has been shown to have a number of cardiovascular effects in human beings. However, neither ingestion nor intra-arterial infusion of ethanol has been shown to affect human heart rate (Ahmed et al., 1973; Tawakol et al., 2004). While melatonin is generally considered a safe supplement, with only rare and mild reported side effects, the result of combining oral melatonin with alcohol is unknown. The FDA strongly advises against the use of alcohol in conjunction with prescription or over-the-counter sleep aids (FDA, 2007). However, individuals with insomnia commonly self medicate with alcohol (Kaneita et al., 2007; Johnson et al., 1998). Melatonin has been shown to decrease heart rate in humans and rats (Gilbert et al., 1999; Chuang et al., 1993), but the combined effect of melatonin and ethanol on heart functions has not yet been studied.

Because melatonin is known to exert a soporific effect, possibly through the modulation of cholinergic transmission, we predict that melatonin will slow the heart rate of *D. magna* by increasing ACh activity in the inhibitory regulatory neurons. We predict that ethanol will increase the heart rate of *D. magna* by inhibiting ACh release. Melatonin and ethanol combined should exert antagonistic effects on the cholinergic system, resulting in a minimal change in the heart rate.

**Materials and Methods**

*D. magna* (WARD’S Natural Science, Rochester, NY) were maintained in jars of spring water under a 130-Watt incandescent bulb. Individual animals were transferred in a drop of water to a depression slide coated in petroleum jelly. The heart rate was counted after a 30-second acclimation period using a Nikon SMZ645 dissecting microscope with a NI-150 High Intensity Illuminator (Nikon Inc., Melville, NY). For the water controls, multiple heart rate readings were taken per flea, starting 30 s after immobilization and continuing for 2-min intervals until a total of five counts had been
completed. The animal was then discarded. A total of 70 control heart rate readings from 14 individual fleas were collected in spring water. For every other condition, only one average heart rate was obtained per flea.

**Effect of Melatonin on Heart Rate**

Powdered melatonin (Sigma Aldrich, St. Louis, MO) was dissolved in deionized water to make a 10 mg/L solution, corresponding to the maximum solubility of the melatonin powder. This solution was stored at 4°C and protected from light due to its photosensitivity. The average heart rate following 0-, 15-, 30-, 120-, and 180-min exposure to melatonin was determined. Individual animals were immobilized on depression slides in a drop of water. The water was then withdrawn and replaced with room temperature melatonin solution. Heart rate was assessed after 0, 15, and 30 minutes had elapsed. For the 120- and 180-min time points, individuals were allowed to swim freely in room-temperature melatonin solution in the dark before the heart rate was scored using the procedure described above. This permitted examination of heart rate at greater time intervals while avoiding any long-term negative effects of immobilization in petroleum jelly.

The maximum effect of melatonin on heart rate was quantified using a 120-min exposure time (based on time lapse trial results, Figure 1). Individual *D. magna* were treated for two hours with melatonin solution as described previously. After immobilization on a petroleum jelly-coated slide, the melatonin solution was withdrawn and replaced with a 5% ethanol/10 mg/L melatonin solution. Heart rate was then assessed after six minutes of exposure to the ethanol/melatonin solution.

**Effect of Ethanol on Heart Rate**

To test the effect of ethanol on heart rate, fleas were immobilized as previously described, and an initial heart rate was counted in water. All water was withdrawn from the slide and quickly replaced with 100 µL of 5% ethanol (EM Science, Gibbstown, NJ), which was the lowest concentration found to elicit a strong physiological response in preliminary testing (data not shown). The heart rate was counted in two minute intervals for a total of ten minutes to determine when the maximal response would occur. Additional trials were then performed using a six-minute exposure time to quantify the maximum effect of ethanol on heart rate.

**Combined Effect of Alcohol and Melatonin on Heart Rate**

Individual *D. magna* were treated for two hours with melatonin solution as described previously. After immobilization on a petroleum jelly-coated slide, the melatonin solution was withdrawn and replaced with a 5% ethanol/10 mg/L melatonin solution. Heart rate was then assessed after six minutes of exposure to the ethanol/melatonin solution.

**Statistical Analysis**

Results were analyzed with either one-way ANOVA followed by Tukey HSD post-hoc comparison (family error rate = 5%) or Dunnett’s method for comparison with control or a Student’s t-test with an alpha level of 0.05 using Minitab15.1.0.0 for Windows (LEAD Technologies Inc., Haddonfield, NJ). All error bars represent the standard error of the mean.

**Results**

A significant decrease in heart rate was observed in response to 10 mg/L melatonin solution only at the 120-min time point (Figure
Effects of Melatonin and Ethanol on the Heart Rate of Daphnia magna

Animals kept in dark conditions for two hours did not exhibit a decrease in heart rate (244 ± 7 bpm) in comparison with controls (228 ± 2 bpm, one-tailed t-test, t(23) = 2.14, p = 0.978).

D. magna exhibited a significant decrease in heart rate immediately following the addition of 5% ethanol, and the heart rate remained significantly depressed over the 10-min period (Dunnett’s Test, α=0.05). The heart rate did not change significantly between two and ten minutes (one-way ANOVA, F=0.08, p=0.989). For logistical reasons, a six-minute exposure time was used to assess the maximum effect of ethanol on heart rate (reported in Figure 3), although the data indicate that any time between two and ten minutes could have been used.

The maximum depressive effects of 10 mg/L melatonin and 5% ethanol on the heart rate were assessed using 120- and 6-min exposure times, respectively. Treatment with ethanol (176 ± 10 bpm, T= -5.51, p<0.00005), melatonin (167 ± 8 bpm, T= -6.75, p<0.00005), and ethanol and melatonin together (89 ± 7 bpm, T= -14.60, p<0.00005) were all found to decrease the heart rate significantly compared to controls (Dunnett’s Test). Treatment means were compared using Tukey HSD post hoc comparisons. Ethanol and melatonin were found to cause identical decreases in heart rate compared to animals in spring water (T= 0.939, p= 0.7838). Six-min ethanol exposure following two-hour immersion in melatonin, however, resulted in a greater decrease in heart rate than either ethanol (T= -8.591, p<0.00005) or melatonin (T= 7.977, p<0.00005) treatment alone (Figure 3).
Immersing *D. magna* in melatonin for two hours caused a significant decrease in heart rate afterward. This depressive effect on heart rate has been demonstrated previously in human beings (Gilbert et al., 1999). Melatonin likely exerts its effects on the water flea heart by altering ACh levels. Application of ACh inhibits the heart rate of *D. magna*, and melatonin has been shown to increase ACh secretion in the rat brain (Baylor, 1942; Paredes et al., 1999; Brusco et al., 1998). It is therefore likely that the observed decrease in the *D. magna* heart rate upon melatonin administration occurs due to modulation of the activity of the inhibitory cholinergic neurons that regulate the heart, most likely through an increase in ACh release. Alternatively, melatonin may affect the heart by altering the binding properties of neuronal nicotinic AChR (Markus et al., 1996; Zago and Markus, 1999). Melatonin receptors have also been identified in the vasculature, so it is possible that melatonin has a direct effect on the cardiovascular system that does not involve modulation of cholinergic activity (Ekmekcioglu et al., 2003).

Immersion in 5% ethanol was shown to decrease the heart rate significantly in *D. magna* after a six-minute exposure time (Figure 2). Similarly, ethanol has been previously shown to slow the heart rate of the invertebrate *Ciona intestinalis* (Pope and Rowley, 2002). The mechanism by which this occurs is uncertain. Ethanol has been shown to have a wide range of effects on physiological function. Ethanol is known to elicit a biphasic response, with stimulatory effects produced at lower doses and inhibitory effects at higher doses (Earleywine and Martin, 1992). The high concentration used in this study would be expected to result in an inhibitory effect. Previously, ethanol has been shown to inhibit the release of neurotransmitters, including ACh, in rats (Carmichael and Israel, 1975; Erickson and Graham, 1973). Application of ethanol, which would inhibit ACh release and therefore decrease cholinergic transmission in these inhibitory neurons, would thus be expected to increase the heart rate of *D. magna*. It is possible that the effect observed in *D. magna* is not due to changes in the release of ACh, but rather in receptor sensitivity to ACh. Ethanol has also been shown to alter the properties of neuronal nicotinic AChR, potentiating ACh-induced currents (Cardoso et al., 1999; Aistrop et al., 1999; Wu et al., 1994). Thus, ethanol may slow the heart rate by acting on AChRs located either on the heart itself or in the chain of regulatory neurons. Ethanol may also act through an entirely different mechanism to cause direct effects on the cardiac muscle of *D. magna*. Ethanol has been shown to impair myocardial contractility in the human heart (Ahmed et al., 1973), and to decrease heart rate in the isolated rat heart (Pagala et al., 1994).

The application of ethanol following a two-hour exposure to melatonin was shown to cause a greater decrease in heart rate than either ethanol or melatonin alone. If melatonin causes
an increase in cholinergic activity, and ethanol inhibits the release of ACh, these two drugs would be expected to have antagonistic effects. Due to their opposing effects on the cholinergic system, these results suggest that ethanol and melatonin act through different mechanisms to influence the activity of the *D. magna* heart. The high concentration of ethanol may have severely impaired the myogenic cells of the heart, and pre-treatment with melatonin may have altered the activity of the cholinergic neurons such that their normal regulatory activity was hindered, causing a more severe depression of the heart rate. According to this mechanism, melatonin may make it more difficult for the regulatory cholinergic system to respond appropriately to stress on the heart (such as the administration of an acute dose of alcohol). Alternatively, the combined effect of alcohol and melatonin could be the result of different actions on the cholinergic system. Melatonin may increase the release of ACh, while ethanol may alter the sensitivity of AChRs. According to this mechanism, ethanol would enhance the response of the *D. magna* heart to ACh, which is elevated by melatonin, to cause the enhanced depression of heart rate. A possible limitation to this study involved the use of petroleum jelly to immobilize the fleas so that heart rate could be counted. Although the heart rate was scored in this manner for all conditions, it is possible that this technique resulted in additional stress for the animals, which may have impacted their response to the drugs. More studies are needed to elucidate the mechanisms by which these two drugs influence the heart and nervous system of *D. magna*.

These results suggest that combining ethanol and melatonin may cause a potentially unsafe decrease in heart rate. This is of concern due to the tendency of those with sleep disorders to self-medicate with alcohol. Melatonin is increasing in popular use as a natural alternative to prescription sleep aids, and due to the lack of strong warning labels, individuals may be less cautious about concomitant alcohol use. More studies are needed to investigate the effect of these two drugs in combination on the mammalian heart. If these additive effects prove to be significant in higher organisms, it may be prudent to include an alcohol warning on melatonin supplements similar to that included with prescription sleep aids.

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