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Article

Nitric Oxide Synthesis/Pathway Inhibitors in *Daphnia magna* Reverse Alcohol-Induced Heart Rate Decrease

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SUMMARY

Studies show that alcohol causes oxidative stress and heart rate (HR) depression in humans. Chronic alcohol consumption also causes cardiac myopathy, which afflicts about 500,000 Americans annually. The present study aims to understand how alcohol reduces HR and examine if select chemical agents with inhibitory effects on nitric oxide levels can reverse the effects of alcohol on HR. In this study, we used *Daphnia magna,* a water crustacean with a large and a transparent heart, as an in vitro model to study the effects of agents such as methylene blue (a guanylyl cyclase inhibitor), melatonin (a free radical scavenger and Phosphodiesterase E5 (PDE5) inhibitor), L-glutamate (N-methyl-D-Aspartate (NMDA)-ion channel stimulator), and L-N(gamma)-nitroarginine methyl ester (L-NAME, nitric oxide synthase inhibitor) on HR by alcohol. We also examined the ability of these agents to prevent a decrease in the HR by alcohol by changing the order in which we exposed the *Daphnia* to the substances. Alcohol exposure decreased HR by 27% after 30 minutes of exposure. The experiment measured the ability of various drugs to reverse this decrease in HR. Methylene blue, after 10 minutes of exposure, increased the HR by 15%. Melatonin, after 10 minutes exposure, increased the HR by 27%. Similarly, L-NAME and L-glutamate, after 10 minutes of exposure, increased the HR by 20% and 36%, respectively. These results showed that alcohol's depressive effects on HR could be mediated through nitric oxide generation and confirmed the hypothesis that nitric oxide synthesis/pathway inhibitors can reverse alcohol-induced HR decrease in *Daphnia magna.*

INTRODUCTION

Alcohol is the most frequently used drug in our society. Alcohol consumption leads to both short-term and long-term cardiac health effects in humans, which are thought to be mediated primarily through oxidative damage caused by reactive oxygen species (ROS), promoting cell injury and death (1). Short-term effects of ethanol decrease HR and P-R interval (onset of P wave to the start of the QRS complex) in rats, whereas in humans, ethanol was reported to decrease myocardial contraction, possibly due to compensatory mechanisms involved (2, 3). The described long-term effects in humans include decrease in HR, eventually leading to cardiac myopathy and heart failure. How alcohol mediates these effects is not well understood.

Alcohol mediates its central nervous system (CNS) effects through altering cell membrane structure, decreasing Ca^{2+} ion currents, and binding to ligand-gated ion channels (4-6). Other reported effects of alcohol include direct effects on membrane receptors and ROS generation (7, 8). Researchers have shown that alcohol depresses several antioxidant enzymes such as catalase and superoxide dismutase, and that it increases free radical content in a rat model (9). The above effects contribute to oxidative stress and possible auto-oxidation of catecholamines involved in HR regulation. Alcohol also exerts inhibitory effects on the CNS through gamma-aminobutyric acid (GABA) receptors and increases in nitric oxide (NO), which have been shown to have depressive effects on both contractile force and HR (10, 11).

Among the above mechanisms of alcohol discussed, the role of free radicals, ligand channels, and nitric oxide are less well understood in *Daphnia*'s HR regulation. The present study explored if alcohol's depressive effects on the HR in *Daphnia* are mediated via the NO pathway, using NO inhibitors. We investigated the ability of select chemicals to prevent or reverse alcohol-induced depressive effects on HR. Specifically, we tested methylene blue (guanylyl cyclase inhibitor), melatonin (increases PDE5 phosphorylation), L-Glutamine (NMDA ion channel stimulator) and L-NAME (NO synthase inhibitor), which are reported in the literature to interfere with free radicals, ion channels (NMDA, Ca^{2+}), or the NO pathway.

In the present study, *Daphnia magna* were an in vitro model to study the effects of some chemicals on heart rate. *Daphnia magna* is a water crustacean, and it is used frequently because of its transparent exterior and its visible internal organs, which allowed us to easily monitor the HR. Additionally, the HR of *Daphnia* is inhibited by acetylcholine and potassium, and is stimulated by adrenaline, indicating that the cholinergic and sympathomimetic actions seen in *Daphnia* are similar to those in vertebrates (12). The cardiac effects observed in *Daphnia* were sometimes different from mammals in that adrenaline accelerated cardiac rates only at high concentrations, whereas atropine had a positive chronotropic effect (13). Acetylcholine has similar effects on the heart in *Daphnia* as in mammals (14). These differences do not impact the present study, which aims to investigate agents impacting the NO pathway. The main advantage of

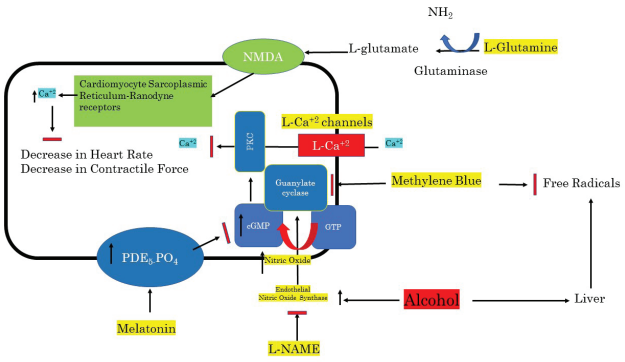

Figure 1: Nitric Oxide-based mechanisms in HR regulation of *Daphnia magna*. The study results suggest a possible therapeutic role for agents that inhibit NO synthesis or signaling pathway in alcohol-induced HR depression. Alcohol increases NO synthesis and cGMP via NO synthase stimulation, which in turn causes a protein kinase cascade to inhibit L-Ca^{2+} channels and Ca^{2+} influx leading to decreased HR and contractile force. Agents which inhibit NO levels directly or indirectly, such as L-NAME (NO synthase inhibitor), melatonin (PDE5 phosphorylation and decreased cGMP), methylene blue (Guanylate cyclase inhibitor) and L-Glutamate (NMDA receptor activator) have resulted in an increased HR in *Daphnia* in this study. All agents reversed the depressive effect of alcohol on the HR and offered protection in post-alcohol treatment. Further investigations are needed to confirm the above proposed mechanisms.

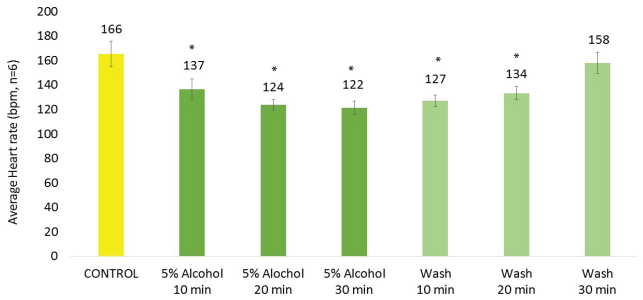

Figure 2: Effect of 5% Alcohol on HR of *Daphnia magna*. *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a Petri dish for up to 30 minutes. HR of Untreated (yellow), 5% alcohol-treated *Daphnia*, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

Daphnia magna as a bioassay is its transparency, which allows easy measurement under a microscope, making them a useful model to study the effects of various agents on the heart (15).

Changes in HR and contractile force are modulated by the extent of calcium flux through L-type calcium channels in cardiac myocytes, which are regulated by a cascade of enzymes and their end products (16). The calcium influx through L-type calcium channels is inhibited by protein kinase G (PKG) activation, which is in turn controlled by cyclic guanosine monophosphate (cGMP), leading to a decrease in HR and contractile force (**Figure 1**). Endothelial nitric oxide synthase (eNOS)-derived NO increases the levels of cGMP via soluble guanylate cyclase (sGC) via synthesis from guanosine triphosphate (GTP). In cardiomyocytes, this stimulates the sGC-cAMP-PKG pathway, resulting in decreased HR (17). Agents L-NAME and methylene blue interfere with this pathway. NO synthesized by neuronal nitric oxide synthase (nNOS) is coupled to ryanodine receptors in the myocardial sarcoplasmic reticulum and elicits increased calcium influx and increased HR and contractile force, which are opposite to the effects of eNOS-derived NO (17). The NMDA receptor agonist L-Glutamine promotes this activity. Finally, melatonin promotes PDE5, leading to decreased cGMP levels, increased calcium influx, and increased HR (19). Collective use of these agents would allow us to confirm involvement of the NO pathway in *Daphnia magna* HR regulation.

RESULTS

Alcohol causes a dose-dependent (1%-5% v/v) decrease in HR in *Daphnia*. In order to determine the alcohol concentration to be used in the experiments, the effect of 5% (v/v) alcohol was measured after 10, 20, and 30 minutes of exposure to alcohol (**Figure 2**). Treatment with 5% v/v alcohol reduced the HR from 166 ± 10 bpm in the control group to 122 ± 5 bpm (27% of control, *p*-value 0.001)) by 30 minutes (**Figure 2**). There was no further decrease in the HR between 20 and 30 minutes (124 ± 4 bpm and 122 ± 5 bpm, respectively). Therefore, 30 minutes was chosen as an end time to measure the effect of 5% (v/v) alcohol on HR, when the HR has reached a plateau. We chose the 5% v/v alcohol

Table 1: Heart rate comparison of water wash and post-alcohol treatment groups at 10 min and 20 mins (bpm ± std.dev)

Control	Pre-Treatment	10 min	20 min	30 min	Post-Treatment	10 min	20 min	30 min
Treatment					Post-Alcohol Treatment			
166 ± 10	Alcohol (5%)	137 ± 8	124 ± 4	122 ± 5	Water Wash	127 ± 5*	134 ± 5*	158 ± 9
170 ± 6	Alcohol (5%)	146 ± 7	140 ± 7	125 ± 9	Melatonin	159 ± 5**	161 ± 13***	150 ± 12
158 ± 3	Alcohol (5%)	132 ± 7	128 ± 4	123 ± 4	L-Glutamine	166 ± 9**	160 ± 10***	158 ± 3
150 ± 5	Alcohol (5%)	143 ± 5	133 ± 6	122 ± 5	L-NAME	146 ± 9**	148 ± 6***	149 ± 5
166 ± 6	Alcohol (5%)	156 ± 4	141 ± 6	128 ± 4	Methylene Blue	147 ± 3**	152 ± 3***	161 ± 3

* Significance of $p < 0.05$ when compared to Control group (166 ± 10 bpm)
** Significance of $p < 0.05$ when compared to water wash group (127 ± 5 bpm)
*** Significance of $p < 0.05$ when compared to water wash group (134 ± 5 bpm)

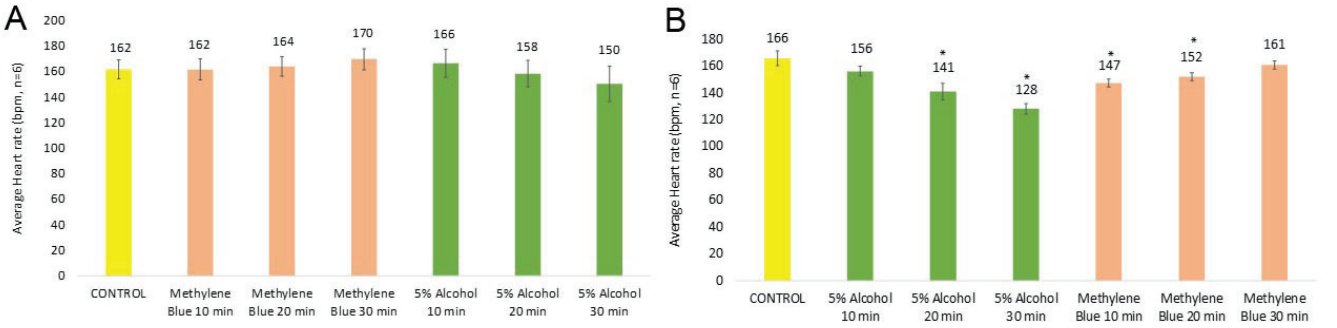

Figure 3: Effect of Methylene Blue Treatment on HR of *Daphnia magna*. A) *Daphnia* were exposed (n = 6) to 100 µL of methylene blue (10 µg/mL) solution in a Petri dish for up to 30 minutes. HR of Untreated (yellow), methylene blue-treated *Daphnia* before alcohol exposure, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). **B)** *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a petri dish for up to 30 minutes. HR of Untreated (yellow), alcohol-treated *Daphnia*, methylene blue-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

concentration because it reduced the HR substantially enough (27% of controls) to allow the visual measurement of reversal effects of chemical agents on HR without causing excessive depression or death.

In order to determine the reversibility of the alcohol effect on HR, the *Daphnia* were washed with 1 mL of water and the effect of wash time was observed every 10 minutes for 30 minutes. The depressive effect of alcohol on HR was maintained until 20 minutes (134 ± 5 bpm, *p*-value 0.001 from the control value of 166 ± 10 bpm). By the end of 30 minutes the HR was 158 ± 9 bpm, or 95% of control (**Figure 2**). The HR values were analyzed at 10 and 20 minutes after the water wash and compared to the HR before the wash (i.e., 30 minutes of alcohol treatment) with a significance threshold of $\alpha = 0.05$. This analysis indicated that the HR values at 10 minutes and 20 minutes post-wash were significantly different compared to the control group (166 ± 9 bpm, **Table 1**, *p*-value 0.001). Additionally, we compared the HR values at 10 and 20 minutes of treatment with each of the agents, which were added after 30 minutes of alcohol exposure, to the corresponding HR values with water wash treatment alone and were found to be significantly different ($p<0.05$, **Table 1**). These data indicated that the HR reversal effects of these agents were more pronounced with a quick onset than the water wash effects and could be measured up to 20 minutes. As a conservative measure we measured all reversal effects of chemical agents by 10 minutes.

Select chemical agents which interfere with NO synthetic pathway were used to examine if NO pathway was involved in alcohol induced depressive effects on HR and whether these effects can be prevented or reversed. All chemical agents were screened in a pre-alcohol exposure experiment for selecting a final concentration based on their toxicity levels and concentration where effects were measurable. Methylene blue (MB) by itself at 10 µg/mL did not have any significant effect ($\alpha = 0.05$) on HR over 30 minutes of exposure, when added before alcohol (**Figure 3A**). Pre-alcohol addition of MB for 30 minutes protected from alcohol-induced HR reduction up to 30 minutes (150 ± 14 bpm, 93% of control), as no significant decrease from control values (162

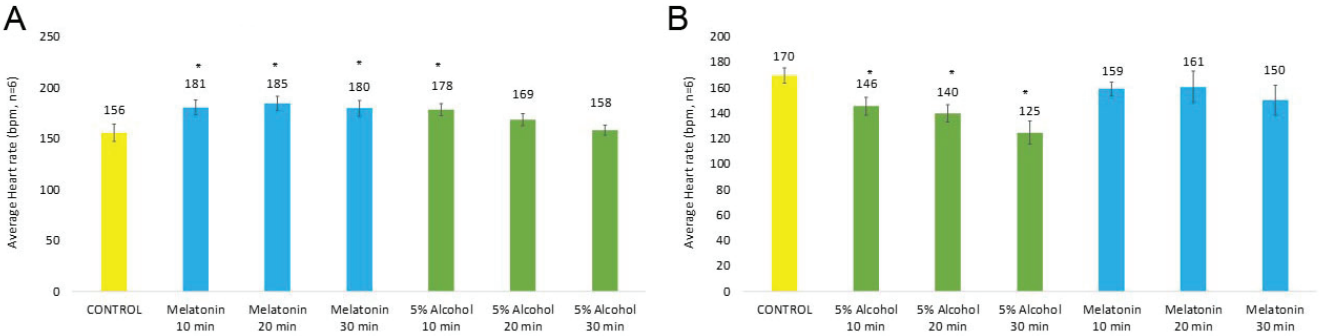

Figure 4: Effect of Melatonin Treatment on HR of *Daphnia magna*. A) *Daphnia* were exposed (n = 6) to 100 µL of melatonin (5 mg/mL) solution in a petri dish for up to 30 minutes. HR of Untreated (yellow), melatonin-treated *Daphnia* before alcohol exposure, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). **B)** *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a petri dish for up to 30 minutes. HR of untreated (yellow), alcohol-treated *Daphnia*, melatonin-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

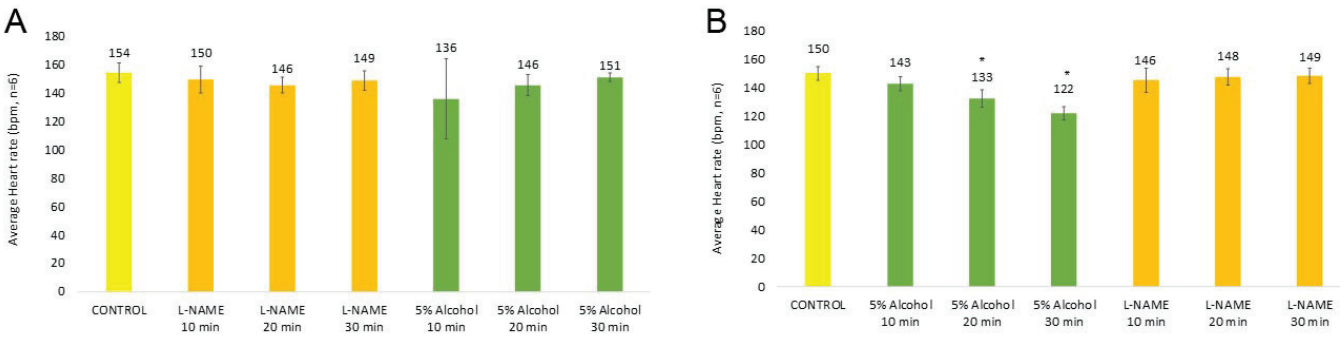

Figure 5: Effect of L-NAME Treatment on HR of *Daphnia magna*. A) *Daphnia* were exposed (n = 6) to 100 µL of L-NAME (3 mg/mL) solution in a petri dish for up to 30 minutes. HR of Untreated (yellow), L-NAME-treated *Daphnia* before alcohol exposure, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). **B)** *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a petri dish for up to 30 minutes. HR of untreated (yellow), alcohol-treated *Daphnia*, L-NAME-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

± 7 bpm) were observed. We next added MB after 30 minutes of alcohol exposure, and by 10 minutes, it had HR increased the HR to 147 ± 3 bpm (90% of control HR of 166 ± 6 bpm, *p*-value 0.001) from the previous depressed rate of 128 ± 4 bpm (**Figure 3B**). These results suggest that MB was able to both prevent and reverse alcohol-induced depressive effects on HR.

Treatment with melatonin alone at 5 mg/mL increased the HR from the control value (156 ± 9 bpm) to 180 ± 8 bpm (115% of control) by 30 minutes of treatment (**Figure 4A**). After 30 minutes of alcohol exposure, the HR decreased to 158 ± 5 bpm (101% of control). Melatonin thus protected from the alcohol-induced decrease in the HR. We next added melatonin after 30 minutes of alcohol treatment, and by 10 minutes, the melatonin had increased the HR to 159 ± 5 bpm (94% control HR of 170 ± 6 bpm, *p*-value 0.009) from the previous depressed value of 125 ± 9 bpm (**Figure 4B**). These results suggest that melatonin was able to counter the alcohol-induced decrease in the HR.

L-NAME treatment at 3 mg/mL resulted in a HR of 149 ± 7 bpm by 30 minutes, which is not statistically different from the control HR of 154 ± 7 bpm (**Figure 5A**). With subsequent alcohol treatment, the HR remained unchanged at 151 ± 3 bpm (98% of control HR) by 30 minutes, indicating that L-NAME was able to protect against the alcohol-induced decrease in HR. After 30 minutes of alcohol pre-treatment, we added L-NAME, and the treatment increased the HR within 10 minutes to 146 ± 9 bpm (97% of control HR, *p*-value 0.266) from a previous value of 122 ± 5 bpm (**Figure 5B**). These results suggest that L-NAME was able to both prevent and reverse the alcohol-induced depressive effects on HR.

Thirty minutes of L-Glutamine treatment alone resulted in a HR increase to 177 ± 6 bpm, a 110% increase from a control HR of 160 ± 3 bpm (**Figure 6A**). When alcohol was subsequently added, the HR decreased to 134 ± 26 bpm (83% of control HR) by 30 minutes, suggesting that L-glutamine could not prevent alcohol's effects. However, treating with L-Glutamine for 10 minutes following 30 minutes of alcohol exposure, increased the HR to 166 ± 9 bpm (105% of control 158 ± 3 bpm, *p*-value 0.092) from a previous value of 123 ± 4 bpm (**Figure 6B**). These results suggest that L-Glutamine was only able to reverse alcohol-induced depressive effects on HR, but it could not protect against the alcohol effects.

DISCUSSION

In the present study, exposure to 5% (v/v) alcohol decreased *Daphnia* HR, approximately by 27%, from 166 ± 10

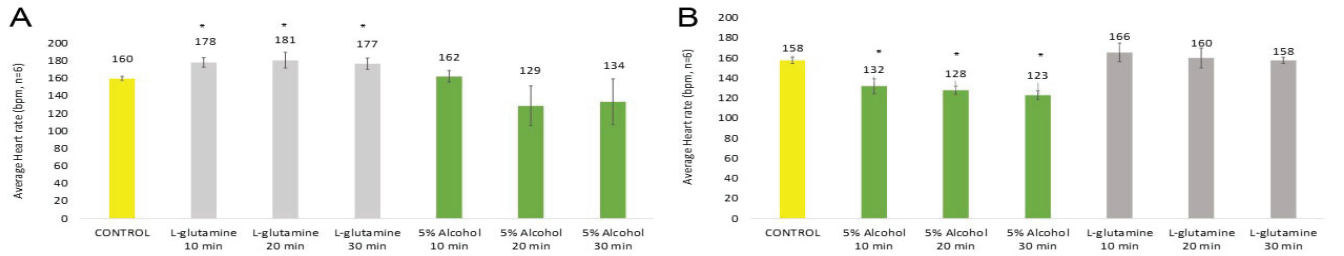

Figure 6: Effect of L-Glutamine Treatment on HR of *Daphnia magna*. A) *Daphnia* were exposed (n = 6) to 100 µL of L-Glutamine (10 mg/mL) solution in a petri dish for up to 30 minutes. HR of Untreated (yellow), L-NAME-treated *Daphnia* before alcohol exposure, alcohol-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). **B)** *Daphnia* were exposed (n = 6) to 100 µL of 5% alcohol solution in a petri dish for up to 30 minutes. HR of untreated (yellow), alcohol-treated *Daphnia*, L-NAME-treated *Daphnia* after water washout, were measured in 10-minute intervals for 30 minutes (n = 6 for each condition, excluding control). Error bars represent standard deviation, and asterisks (*) represent statistical significance compared against control HR (student's t-test, *p*-value < 0.05). Control bars, alcohol bars are shown in yellow and green colors respectively.

bpm to 122 ± 5 bpm in controls by 30 minutes (**Figure 2**). The reversal of this decrease was monitored as a parameter in this study to understand if alcohol mediates its effects through the NO pathway with the use of select inhibitors of enzymes — nitric oxide synthase (L-NAME) and sGC (methylene blue). Additionally, the ability of two other agents, one acting through the NMDA receptors (L-Glutamine) and the other via phosphodiesterase 5 (melatonin), on cGMP levels were studied for their ability to reverse alcohol-induced depressive effects on the HR.

Methylene blue by itself did not have any significant effect ($\alpha = 0.05$) on *Daphnia* HR over 30 minutes of exposure (**Figure 3**). Pre-treatment with MB for 30 minutes protected from alcohol-induced HR reduction for up to 30 minutes (150 ± 14 bpm), and no significant decrease from control values (162 ± 7 bpm) was observed. Adding MB for 10 minutes after 30 minutes of alcohol exposure increased the alcohol-depressed HR back up to 147 ± 3 bpm (90% of control HR of 166 ± 6 bpm, p-value < 0.05) from a previous value of 128 ± 4 bpm (**Figure 3A,B**). These effects could be due to MB's inhibitory properties on sGC and the consequent PKG activation (17). L-type calcium channels have been shown to facilitate the action potentials in the atrioventricular node (AV) and contribute to regular HR, and their inhibition by alcohol leads to reduced HR (18). The reversal of alcohol-induced HR depression by MB treatment, as seen in this study, suggests a role for the sGC-cGMP-PKG pathway in alcohol-induced depressive effects on HR.

Melatonin is a free radical scavenger, an antioxidant, and can antagonize nitric oxide activity (19). Melatonin is a hormone produced by the pineal gland and is known to increase the phosphorylation of the enzyme PDE5 four-fold, which in turn decreases the cGMP levels produced by nitric oxide and antagonizes its relaxation effects (20). Exposure to melatonin alone in the pre-alcohol treatment group significantly (p-value < 0.05) increased the HR within 30 minutes to 180 ± 8 bpm from a control HR of 156 ± 9 bpm (**Figure 4A**). Pre-treatment with melatonin for 30 minutes also protected against the alcohol-induced HR reduction up to 30 minutes (158 ± 5 bpm) as no significant decrease from control values (156 ± 9 bpm) was observed. After 30 minutes of alcohol exposure, the subsequent treatment of 5 mg/mL melatonin for 10 minutes increased the alcohol-depressed HR to 159 ± 5 bpm or 94% of control levels (170 ± 6 bpm) in the present study (**Figure 4B**). These protective effects may be the result of the decreased cGMP levels caused by melatonin, which could potentially decrease the PKC and consequent L-type calcium channel activation leading to increased HR (20).

The third agent that was used is L-NAME, which is a well-known non-specific inhibitor of nitric oxide synthase, and it decreases the levels of nitric oxide (21). Sole exposure to L-NAME had no significant ($\alpha = 0.05$) effect on the HR over a 30-minute period (**Figure 5A**). Pre-treatment with L-NAME for 30 minutes protected from alcohol-induced HR reduction up to 30 minutes. After 30 minutes of alcohol exposure, we added 3 mg/mL L-NAME for 10 minutes, which increased the lowered HR from 122 ± 5 bpm to control levels 146 ± 9 bpm (**Figure 5B**). It is possible that alcohol exposure for 30 minutes causes an increase in nitric oxide levels, leading to the activation of the sGC-cGMP-PKC pathway and consequent ligand binding channel (L-channel)/Ca^{2+} influx inhibition (11, 16). This could cause a reduction in the HR. L-NAME, due to its non-selective inhibitory action on nitric oxide synthase could decrease NO production and as a result reverse the inhibitory effects on L-channels/Ca^{2+} influx (21).

The fourth agent used was L-Glutamine, a precursor amino acid for L-glutamate, and L-channel agonist. L-glutamate is formed by deamidation of L-Glutamine by the enzyme glutaminase. L-glutamate is a known agonist for the NMDA ion channels which are a part of the glutamine receptors, which mediate most fast excitatory transmission in the CNS (22). Exposure to L-Glutamine for 30 minutes in the pre-alcohol treatment group increased the HR from 160 ± 3 bpm by 10% to 177 ± 6 bpm (**Figure 6A**). However, pre-treatment with L-Glutamine for 30 minutes did not protect from the effects of alcohol-induced HR reduction up to 30 minutes. After 30 minutes of alcohol exposure, the addition of 10 mg/mL L-Glutamine for 10 minutes increased the lowered HR from 123 ± 4 bpm to 166 ± 9 bpm (105% of control level of 158 ± 3 bpm, **Figure 6B**). L-Glutamine binds to the NMDA glutamine receptor, and its activation increases the synthesis of nitric oxide in the CNS by stimulating the nNOS (22). It is possible that NMDA receptor activation and subsequent NO release in cardiomyocytes results in the ryanodine receptor stimulation in the sarcoplasmic reticulum leading to calcium release, muscle contraction, and AV node activation (23). L-Glutamine also has been shown to inhibit nitric oxide formation by inhibiting nitrogen oxide synthase in bovine endothelial cells (24). This effect was proposed via the metabolism of glutamine into glucosamine, which reduces the cellular availability of the NADPH cofactor for nitric oxide synthase, thereby inhibiting its activity. These mechanisms could possibly explain the reversal of alcohol's depressive effects on HR by L-Glutamine.

Overall these results indicate that indirect and direct inhibition of nitric oxide synthesis/pathway by various agents results in the reversal of the alcohol-induced depressive effects on the *Daphnia* HR (**Figure 1**). These data support the hypothesis that alcohol could mediate its depressive effects on the HR via the nitric oxide pathway. Further studies are necessary to confirm the validity of this hypothesis by the measurement of the actual abundance of nitric oxide synthase, soluble guanylate cyclase, phosphodiesterase-5 as well as nitric oxide, and the verification of the involvement of other potential mechanisms which this study did not aim to do. The verification of the validity of these mechanisms in other mammalian organisms is also desirable. The agents which have direct or indirect nitric oxide inhibitory action would have a beneficial therapeutic effect against alcohol-induced

depressive effects on HR.

MATERIALS AND METHODS

Daphnia magna were ordered from Carolina Biological Supply (North Carolina, NC). They were stored in loosely lidded glass jars filled with spring water at room temperature. *Daphnia magna* were fed yeast culture every 2-3 days. Drug solutions were freshly prepared at various indicated concentrations in the results section by diluting the stock solutions of methylene blue (1% solution), melatonin (10 mg/mL), L-Glutamine (50 mg/mL), and L-NAME (9 mg/mL) with water in polypropylene test tubes (Fisher scientific). Alcohol (ethanol) solution was prepared at 5 % v/v concentration by dilution with water from 95% v/v.

There were six organisms in each group and their HR (bpm) was measured for 15 seconds and calculated for 1 minute, every 10 minutes up to 30 minutes with a hand counter. The mean and standard deviation were then calculated. There were two types of controls. One control was the HR obtained without any treatment (1st column labelled as control in graphs 2-6. The second control was the effect of the chemical agent by itself on the HR measured in the pre-alcohol cohorts (columns 2-4 in each graph), prior to alcohol administration. Finally, the same organism was used for the control, pre-alcohol or post-alcohol effects measurements, and the results from the replicate experiments (n = 6) were averaged.

All chemical agents were screened in a pre-alcohol exposure experiment for selecting a final concentration based on their toxicity levels and concentration where effects were measurable (data not shown). Methylene blue (half maximal effective concentration (EC50) in *Daphnia* ~ 2.28 µg/mL) was tested at 1.1, 3.3, and 10 µg/mL concentration levels (25). At 1.1 µg/mL MB was not effective in preventing alcohol-induced depression, but was from 3.3 µg/mL and higher. The 10 µg/mL concentration was chosen as the effects were more pronounced. Melatonin (median lethal dose for 50% of the animals (LD50) in rats > 800 mg/kg) was tested at 5.0, 1.0, and 0.33 mg/mL concentration levels (26). Melatonin protected from alcohol-induced depressive effects at 1 mg/mL concentration and higher, and a 5 mg/mL concentration was finally chosen. L-NAME (LD50 not available) was tested at 3, 1, and 0.33 mg/mL concentration levels. L-NAME did not protect from alcohol-induced depressive effects at 0.33 and 1.0 mg/mL concentrations, and a 3 mg/mL final concentration was thus chosen. L-glutamine (LD50 in rats ~ 7.5 gm/kg) was tested at 10, 3.3, and 1.1 mg/mL concentrations (27). L-glutamine was not effective at 3.3 mg/mL and below in protecting from alcohol-induced depressive effects, and so a 10 mg/mL final concentration was chosen.

Daphnia was placed in the wells of concave slides using a Nalgene dropper with a wide mouth (provided by Carolina Biologicals) and were given 10 minutes to acclimate (**Figure 7**). The surrounding water was slowly aspirated with the dropper just enough to restrict the mobility of the organism,

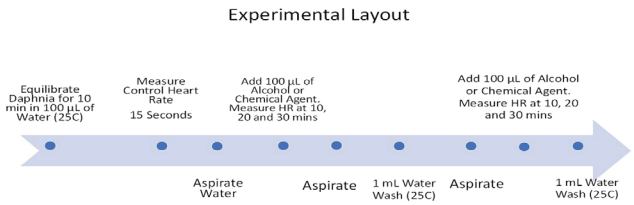

Figure 7: The experimental layout depicts the procedures of equilibration, wash and addition of agents to *Daphnia*. After equilibration at room temperature, control *Daphnia* HR (n=6) is measured and either alcohol or a chemical agent is added. HR is monitored at 10-minute intervals for 30 minutes. After washout with water, the treatment in the previous step is reversed, and the HR is monitored at 10-minute interval for 30 minutes.

and the slide was placed under a digital microscope connected to a personal computer. The HRs were measured for 15 seconds using a timer (Fischer Scientific), and the total beats were calculated per minute, and this was considered the control HR. The surrounding water was then completely aspirated, and 0.1 mL of alcohol solution (5% v/v) was then added. The HRs were again measured for 15 seconds for every 10 minutes, up to 30 minutes. The alcohol solution was then aspirated with a dropper, prior to the HR measurement for 15 seconds and added back to resume the experiment until 30 minutes. Then the alcohol solution was completely aspirated, and the *Daphnia* were washed with 1 mL of water. The wash liquid was completely aspirated, and 0.1 mL of chemical agent solution prepared in water was then added. After 10 minutes equilibration, the solution was aspirated, and the HRs were measured for 15 seconds (for every 10 minutes), and the solution was added back to resume the exposure for 30 minutes. This process was repeated for each drug, using a total of six *Daphnia* for each trial of pre- or post-alcohol exposure. Pre- and post-alcohol exposure sets used different sets of *Daphnia*, and each *Daphnia* once used was never used for any other experimentation.

Chemical agents were added, either before or after the alcohol exposure for 30 minutes for all treatment groups. If chemical agent treatment was after the alcohol treatment (post-alcohol treatment), then the ability of the chemical agent to reverse the decreased HR is observed. If chemical agent treatment was before the treatment with alcohol (pre-alcohol treatment), then the ability of chemical agent to prevent the drop in the HR by alcohol is looked for. All alcohol treatments were performed up to 30 minutes, and the reversal effects of the agents were measured for 30 minutes, but only results at 10 minutes were considered for comparison.

Statistical Analysis

HRs were obtained at 15 second intervals and were multiplied with 4 to calculate the total beats per minute (bpm). The average HR (n = 6) for a group and the standard deviation values were calculated using Microsoft Excel. A student's t-test was also conducted to find the differences between

the control group and drug-treated groups. This was done by determining the *p*-values (at a significance level of 0.05), which showed that the data for drug-treated groups was significantly different from that of the controls.

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