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Preference for ethanol in zebrafish following a single exposure

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Abstract

Ethanol is one of the most widely abused drugs in the world. Its addictive property is believed to primarily stem from its ability to influence the brain reinforcement pathway evolved for mediating natural rewards. Although dopamine is a known component of the reinforcement pathway, clear molecular and cellular compositions of this pathway and its sensitivity to ethanol remain not well understood. Zebrafish has been increasingly used to model and understand human disease states, due to its genetic tractability and ease of maintenance. In this study, we determine whether adult zebrafish develop ethanol preference after a single exposure using a conditioned place preference (CPP) paradigm. Moreover, we establish a procedure that can be carried out in an automated and relatively high-throughput fashion. We find that zebrafish of the AB strain display significantly increased preference for the compartment where they received ethanol during a single 20 -minute exposure. The largest increase in preference is in response to a 1.5% ethanol administered in the tank water. The results demonstrate robust ethanol preference in zebrafish. Such a relatively high-throughput assay with automated tracking and response to a single ethanol exposure provides a potential means for a large-scale screening aimed at understanding the brain reinforcement pathway and its sensitivity to ethanol in this genetically tractable vertebrate.

Keywords

Ethanol; zebrafish; conditioned place preference (CPP); reinforcement pathway; addiction; reward

2. INTRODUCTION

Alcoholism is a disease which involves craving, loss of control, physical dependence and tolerance (Diamond & Gordon 1997, Lê et al. 2001). Alcohol abuse and alcoholism cut across gender, race, and nationality. In the United States, 17.6 million people--about 1 in every 12 adults--abuse alcohol or are alcohol dependent (Sources: NIAAA 2007). It is not yet clear why certain individuals are more susceptible to alcohol addiction than others. A better understanding of the molecular and cellular mechanisms underlying the biological effects of ethanol shall provide important insights into understanding and treating human conditions.

Zebrafish is a vertebrate model organism that is amenable to genetic manipulation (Grunwald & Eisen 2002, Guo 2004) and to studying the effects of drugs on behavior (Guo

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2009). Larval zebrafish have been proposed as a tractable vertebrate model system for a large-scale genetic analysis of the biological effects of alcohol (Lockwood, Bjerke, Kobayashi, & Guo 2004). Using such a system in an ethanol-modulated camouflage response screen, important signaling molecules have been identified that regulate acute locomotor behavioral sensitivity to ethanol (Peng et al. 2009). Some basic behavioral tests to investigate the acute effects of alcohol on adult zebrafish have been proposed (Gerlai, Lahav, Guo, & Rosenthal 2000). Effects of acute and chronic ethanol administration on startle reaction, area occupied by a group of subjects and the average distance between each fish and its nearest neighbor have been investigated in three strains of zebrafish (Dlugos & Rabin 2003). In another study, ethanol administration was found to ameliorate predator avoidance, a potential anxiolytic effect (Gerlai, Lee, & Blaser 2006). Additionally, ethanol treatment was found to result in significant dose-dependent behavioral changes but the dose-response trajectories were found to differ among zebrafish populations demonstrating a genetic component in alcohol responses in adult zebrafish (Gerlai, Ahmad, & Prajapati 2008).

One prominent feature that distinguishes drugs of abuse (e.g. ethanol) and non-addictive substances is that drugs of abuse have the powerful ability to reinforce behaviors. It is generally thought that they somehow “tap” into the same neural systems that mediate natural rewards (Kelley & Berridge 2002). Studies in animal models (mainly rodents) indicate that most drugs of abuse activate dopaminergic (DA) neurons either directly or indirectly (Di Chiara & Imperato 1988), thus implicating the dopamine system as an important component of the brain reinforcement pathway. Despite these advancements, the molecular and cellular compositions of the reinforcement pathway and its sensitivity to ethanol are not well understood.

Conditioned place preference (CPP), a behavioral paradigm in which a rewarding substance is paired repeatedly with an environmental cue so that the animal associates the cue with the rewarding properties of the substance and in turn develops a preference for the cue, has been extensively used to study the reinforcing properties of drugs of abuse in rodents (Tzschentke 1998, T.M. Tzschentke 2007). In zebrafish, CPP for the natural reward food, and addictive drugs including cocaine, morphine, and amphetamine has been demonstrated (Darland & Dowling 2001, Lau, Bretau, Huang, Lin, & Guo 2006, Ninkovic et al. 2006).

CPP to ethanol has been extensively described in rats and mice (Tzschentke 1998, T.M. Tzschentke 2007), but so far there is only one report of CPP response to ethanol in adult zebrafish (of Tuebingen genetic background) using manual tracking (Kily et al. 2008). Given that both CPP and conditioned place aversion (CPA) have been observed in rodents treated with ethanol, suggesting that methodological and procedural parameters greatly influence the outcome of conditioning (Tzschentke 1998, T.M. Tzschentke 2007), it remains uncertain, based on a single study, whether ethanol truly induces preference in zebrafish across different genetic backgrounds. Thus, we determined whether an ethanol preference could be observed in the wildtype (WT) adult zebrafish (of AB genetic background) using an automated tracking system with potential high-throughput applications. We found that the AB WT zebrafish displayed robust CPP response to ethanol after a single 20 –minute exposure. Moreover, the assay can be carried out in a relatively high throughput manner.

3. METHODS

Rearing of Experimental Animals

A total of 48 zebrafish ranging in age from 6–9 months were used in the present study. Adult zebrafish were maintained and bred following standard procedures (Westerfield 1995). AB is a laboratory-bred strain from the University of Oregon. Adult zebrafish were

fed twice a day with a mixture of live brine shrimps (Platinum-Grade *Artemia* Brine Shrimp, Argent Chemical Laboratories, Redmond, WA) and flake food (Tropical Flakes, Aquatic Eco-Systems, Apopka, FL). They were maintained on a 14/10-h light/dark cycle at 28 deg C. The average body weight for wildtype zebrafish used in our experiments is approximately 0.2 g. All experiments were carried out in accordance with the guidelines laid down by the NIH regarding the care and use of animals for experimental procedures. Only male fish were used for these experiments to reduce variability and these fish were housed singly for at least one day before the experiment.

Ethanol CPP

Rossville Gold Shield® Ethyl Alcohol (Gold Shield Chemical Co. Hayward, CA 94545) was used in this study. Zebrafish were randomly assigned to one of the following four ethanol (EtOH) treatment groups: EtOH 0.00% (v/v) (system water), EtOH 0.50%, EtOH 1.00%, and EtOH 1.50%. The exact sample sizes used in the assays were as follows 0.00% EtOH (n = 8), 0.50% EtOH (n = 11), 1.00% EtOH (n = 11) and 1.50% EtOH (n = 8). Five fish were not included in the study because they displayed an initial preference of greater than 80% for either compartment (2 for the white compartment, and 3 for the dotted compartment). Five fish were excluded from the study because they exhibited freezing behavior for longer than 30 seconds either during initial (n=2) or final preference recording (n=3). Of the three fish that were excluded because of freezing during final recording one had received system water during conditioning, one received 0.5% ethanol and the third had received 1.5% ethanol during conditioning. Freezing behavior appeared to be unrelated to alcohol dose since it was observed during initial recording as well as in fish which received 0.0%, 0.5% and 1.5% ethanol. All experiments were conducted between 10:00 h and 18:00 h in a quiet room maintained at 28 °C. A fan was kept on in the room to provide background noise. The CPP chambers used had the dimensions of 52 cm in length, 16.6 cm in width and 22.9 cm in depth. All chambers were divided into two halves by a 7.6-cm wide brown central alley: one half consisted of white and the other half consisted of dotted patterns. The dotted pattern was made up of 16 dots of 2 cm diameter each in a fixed pattern. The dots were blue in color so that the animals could be automatically tracked by Ethovision even when it swam over the dot. White tape was placed along the edges of the interior of the tank to ensure that the zebrafish could be visualized clearly at the edges. The chamber was placed inside a rectangular brown box insulating the fish from the outside environment. A digital video camera (Sony flash memory digital camcorders SONY Corp. Japan) was placed approximately 1.2 m above each of the four chambers. Each chamber contained four liters of system water (5 g of instant ocean salts and 3 g of sodium bicarbonate per 20 l of water). On day 1 of ethanol CPP experiment, zebrafish were fed in the morning and 2 h later, a single zebrafish was introduced to the middle alley of the CPP chamber for 30 seconds. On the removal of dividers, the animal had free access to all chambers. After habituation of 2 min, its initial preference for white or the dotted environment was assessed through a 5-min recording. Animals that showed an initial strong preference (>80% time spent) for either side were excluded from the study (n=5). Therefore, animals that showed an initial preference between 50.1 and 79.9% for either side were used for subsequent experiments. On Day 2, those that showed an initial preference for the white side were exposed to ethanol on the dotted side: (a) some of these animals were exposed to ethanol first on the dotted side for 20 min, followed by system water on the white side for 20 min and (b) some of the animals received system water first on the white side followed by ethanol on the dotted side. Those that showed an initial preference for the dotted side were exposed to ethanol on the white side with the same balanced procedure as above. In between the ethanol and water exposures fish were placed in individual tanks containing system water for 2 minutes to ensure that all the ethanol was washed off from the body surface of the fish and did not contaminate the tank water in subsequent conditioning sessions. Several identical chambers

were constructed and separate chambers were used for exposing fish to ethanol and system water to avoid contamination. Since dividers were used to restrict the fish to its least preferred side, up to 8 animals could be conditioned simultaneously. During the last minute of the 20-minute conditioning session, a 1 minute recording was made of each fish. This recording was used to determine the swimming speed of fish after being exposed to 0.00%, 0.50%, 1.00% or 1.50% ethanol. Only recordings from the first conditioning session of each fish were used to calculate either basal or ethanol-modulated locomotor activity. Thereafter, the animal was returned to its housing system. Fish were food-deprived for 24 hours before the final preference recording to eliminate variations in response due to variations in the amount of food consumed before the recording. On day 3 (24 h later), the animal was reintroduced into the CPP chamber, and its preference for the white or the dotted side was assessed in an ethanol-free environment through a 5 minute recording after 2 min habituation to the tank. We took a number of precautions to avoid confounding effects due to handling of the fish. Throughout the experiments, care was taken to move fish gently between home tanks and test tanks as well as between home tank and conditioning tank. Moreover both control and ethanol-treated fish were handled the same way. We used dividers and placed the fish in the central alley at the beginning of both initial and final preference recording to avoid placing the fish on the white or dotted compartments. We used light plastic dividers that were removed very smoothly from the top of the tank with minimal disturbance to the fish. The water level inside the tank was just one fifth of the total tank/divider height and the tank was inside the brown box so the fish could not see the experimenter who was removing the dividers. Both dividers were removed simultaneously so the fish were not biased to swim in one particular direction.

Quantification of Behavior

The Noldus MPEG recorder was used to record digital video files simultaneously from four cameras focused on four tanks. The four video recordings could be viewed live simultaneously on a computer screen and were converted to digital AVI files, which could be stored on the hard drive. Before each recording the background image of each tank was recorded and was divided into three arenas (Arena 1 = white, Arena 2 = dotted and Arena 3 = brown middle alley). For each tank the arenas were calibrated by marking the width of the tank in the image and entering its actual value. Ethovision XT version 5.0 (Noldus Info. Tech., Wageningen, The Netherlands), an automated video-tracking method was used to analyze the digital video files as previously described (Blaser & Gerlai 2006). Ethovision tracks the fish as a dark object on a light background. Output from this program allows us to visualize the tracks/pattern of the movement of the fish precisely over a 5 min period and also calculates its velocity/distance covered. Detection threshold levels (minimum pixel changes between background and live images) were set to minimize the effect of debris or reflection. Contrast as well as minimum and maximum sizes of the subject were also set. The pixel cluster within this range was interpreted as the target subject (zebrafish) and the X,Y co-ordinates of the center of the subject were recorded. The following parameters were quantified: a) Distance moved in each arena, b) Velocity in each arena. These two parameters were used to calculate how much time each fish spent on the white and dotted sides before and after conditioning. We used the total duration of recording (5 minutes or 300 seconds) to calculate the percentage of total time spent on white and dotted sides for each fish before and after conditioning. For each fish the side where it received ethanol was noted and change in preference for the conditioned compartment was calculated. The result is expressed as change in percentage preference (% time in the ethanol-paired compartment after conditioning minus the percentage time in the ethanol-paired compartment before conditioning). The control animals were handled exactly the same way as the experimental animals, except that ethanol was omitted during the conditioning trials.

Statistical Analysis

We used Student's t test to assess significant effects when comparing initial preference, change in preference and swimming velocity of fish exposed to system water or various ethanol concentrations. Paired t test was used to assess significant differences between initial and final preference for the conditioned compartment for each group. All tests were carried out using GraphPad Prism Version 5.0.

Measurement of Brain ethanol level

Ethanol absorption was measured using a previously described protocol for measuring internal ethanol concentration in 7 day post fertilization (dpf) larval zebrafish (Lockwood et al. 2004). To prepare samples for the assay, we exposed adult zebrafish to 1.5% ethanol in a 1L tank for 20 minutes. Immediately after exposure and 3 rinses to remove external ethanol, zebrafish were euthanized with 100mg/l tricaine (3-aminobenzoic acid ethyl ester, Sigma). Dissection was carried out under the microscope and the brain was rinsed in cold PBS, blotted with kimwipes and transferred to an eppendorf tube on ice. Ethanol treated brains were pooled together and the average volume of each brain was determined by volume displacement. Each brain was then homogenized individually in 100ul of 50mM Tris-HCl (pH7.4). The homogenate was centrifuged for 20 min at 4 deg C. Samples were processed using an ethanol assay kit (Diagnostic chemicals), which uses the enzyme alcohol dehydrogenase to catalyse the conversion of ethanol to acetaldehyde and NADH. Production of NADH corresponds to an increased absorption at 340 nm, which is proportional to the ethanol present in the sample. We measured the volume of a single adult zebrafish brain and found it to be approximately 10 ul. This volume was used to calculate the millimolar ethanol concentration of the brain. We converted this to % w/v and then to % v/v by dividing with the density of ethanol (0.789 kg/L).

4. RESULTS

In our study, the rewarding effect of ethanol was assessed by the ability of this substance to enhance the animal's preference for an initially non-preferred environment. We used a chamber with three compartments (Fig. 1A): two conditioning compartments which had different visual cues (white vs. dotted patterns) and a third brown-colored center compartment serving as a connection between the two conditioning compartments and as a 'start area' for introducing animals into the CPP chamber. Ethanol CPP experiment was subsequently carried out as described in the Methods section and schematized in Fig. 1B.

Ethanol concentrations of 0.5%, 1%, and 1.5% (v/v in the tank water) were used in this study, based on previous findings, which show that ethanol dissolved in the tank water has demonstrated central nervous system effects in zebrafish, and that the internal ethanol concentrations achieved through these exposures are within the range of what a human drinker would experience (Dlugos & Rabin 2003, Lockwood et al. 2004). Indeed, our own measurement of brain ethanol levels showed that an exposure to 1.5% (v/v) ethanol in the tank water for 20 minutes resulted in a brain ethanol level of ~0.014% (v/v) (Table 1). We explored the possibility of using higher concentrations of ethanol to induce CPP in zebrafish. We found that 20-minute exposure to 2.5% or 3.5% ethanol was lethal to zebrafish: After 10 -minute exposure to such high concentrations, zebrafish showed erratic movements, either freezing at the tank bottom or swimming upside down. Therefore, we conclude that 1.5% ethanol is the highest concentration that is suitable for CPP studies.

Zebrafish displayed no significant preference for the dotted vs. the white compartment initially (Fig. 2). 18 fish preferred the dotted compartment and 20 fish preferred the white compartment. On an average fish spent $11.33 \pm 1.75\%$ of time in the central compartment during initial preference recording.

A typical set of Ethovision -generated automated tracks were shown in Fig. 3. Only the tracks are used by Ethovision to calculate distance moved and velocity. Since the middle alley is brown and does not provide enough contrast, the fish is not always tracked in the middle alley and the tracks sometimes appear to stop at the edge of the white or dotted side. After testing a number of animals and carrying out statistical analysis, we found that, while the control fish and fish treated with 0.5% ethanol showed minimal alteration in their place preference (spending about 40% time in each compartment, and ~20% time in the smaller central alley), fish exposed to 1% ethanol showed a trend of increase in preference and fish exposed to 1.5% ethanol displayed a significant increase in place preference for the drug-paired compartment (Fig. 4A and Fig. 4B). Student's *t* test indicated that 1.5% ethanol produced a significant increase in preference for the conditioned compartment. There was no significant difference in preference for the conditioned compartment whether the fish is conditioned to ethanol first or water first (Fig. 4C).

When percent time spent in the conditioned compartment before and after conditioning with each concentration of ethanol (0%, 0.5%, 1% and 1.5%) was compared, 1.5% ethanol had a significant effect (Paired *t* test, $p = 0.0268$) for before 1.5% ethanol compared with after 1.5% ethanol (Fig. 4A). When change in preference for the conditioned compartment between groups receiving 0%, 0.5%, 1% and 1.5% ethanol was compared, 1.5% ethanol had the most significant effect on change in preference for the conditioned compartment (Student's *t* test, $p = 0.0409$) for 1.5% ethanol compared to 0% ethanol (Fig. 4B). We also performed Student's *t* test and found no significant difference between initial preference values of any of the ethanol-treated groups (0.5%, 1.0% or 1.5%) and the control (0.00% ethanol). Together, these results indicate that adult zebrafish exhibit significant place preference after a single paired exposure to ethanol in a concentration dependent manner.

Ethanol is known to affect locomotor behavior at certain concentrations. We measured swim velocity of fish after 19 -minute exposure to either 0%, 0.5%, 1%, or 1.5% ethanol. Since all fish were exposed to system water (0% ethanol) we were able to collect 24 observations for this group by including all the recordings for which the fish was exposed to water during the first conditioning session. We found that although the animals exposed to 1% or 1.5% ethanol displayed a trend of increased locomotor activity, there was no significant difference in swim velocity of various groups (Fig. 5). These results indicate that ethanol-stimulated locomotor behavior is unlikely to be causally related to ethanol preference in zebrafish.

5. DISCUSSION

In the present study we have established that it is possible to induce CPP in adult zebrafish after a single exposure to ethanol directly administered in the tank water. The establishment of this high throughput and robust assay in zebrafish paves an important way for future screening aimed at identifying genes, cellular networks, or small molecule compounds, which can modulate the reinforcing effect of ethanol.

Although there was no significant effect of conditioning order on change in place preference, we did observe that there is a trend of greater preference when the animal receives ethanol in the second rather than the first conditioning session. The greater preference in the animals receiving ethanol second could be because the animals receiving ethanol first may also experience some residual rewarding effects of ethanol during the second conditioning session (in system water). Thus animals receiving ethanol first may develop a weaker association of the ethanol-paired compartment.

CPP experiments in rodents usually involve a number of lengthy conditioning sessions. Therefore it is of considerable practical interest to employ experimental protocols that reduce the time needed for conducting experiments without reducing the value and

significance of the data (T. M. Tzschentke 2007). A single exposure to the tank was used to establish baseline preference in the present study. (Lau et al., 2006)(Darland and Dowling, 2001) and (Kily et al., 2008) have previously used a single exposure to the CPP tank to establish baseline preference and we also found that repeated exposure to the tank did not significantly alter baseline preference. Furthermore, we showed that a single exposure to ethanol is sufficient to induce CPP. Dlugos and Rabin (2003) have shown that brain alcohol levels in zebrafish can be detected within 15 minutes of exposure and other studies have detected ethanol in the brain of the goldfish after 2 minutes of exposure (Greizerstein & Smith 1973). Thus zebrafish provides the possibility of conditioning with a single exposure to ethanol thereby allowing for a potentially high-throughput screen.

In the only other report of a CPP assay in response to a single exposure of ethanol in zebrafish (Kily et al. 2008), 0.5–1g, 4-month old, sex and age matched Tuebingen wild-type stock were used. The present study used a different strain of fish, the AB strain and these fish were 6-month old male fish. Thus our study shows that in addition to the Tuebingen strain, the AB strain also show a robust CPP response after a single exposure to ethanol. Both studies tested 0.5%, 1% and 1.5% ethanol concentrations (v/v) for 20 minutes. While the previous study found 1% ethanol to be effective, we found that 1.5% ethanol was effective in inducing significant place preference in the present study. These differences could be attributed to the difference in genetic backgrounds, age, or gender of the animals tested.

Another important difference between our study and Kily et al. 2008 is that our study used an automated tracking system, which recorded the position of the fish several times each second and gave us a measure of the actual time spent by each fish on the white or dotted side, while the previous study relied on manual tracking. Since zebrafish can make very quick darting movements, manual tracking is of less accuracy than automated tracking. Moreover, automated tracking allows the assay to be made high throughput. In the present study, automated recording and tracking of movies allows us to simultaneously record initial and final preferences of 4 fish and condition 8 fish at a time. With adjustments of the positions and numbers of tanks and cameras, we can easily increase the number of fish to be simultaneously tracked in the future.

Previous studies in rodents imply that the locomotor stimulatory effect of ethanol may reflect to some extent the reinforcing effect of ethanol (Phillips & Shen 1996). However, in this study, we found that the concentrations and time of exposure of ethanol leading to CPP did not significantly increase the locomotor activity in zebrafish. These observations suggest that the locomotor effect of ethanol is not causally related to the reinforcing effect of ethanol. Although some common neural substrates such as dopamine, GABA, serotonin, and glutamate have been implicated in both ethanol-modulated locomotor behaviors and ethanol preference, certain components of the neural circuitries mediating these behaviors are likely to be distinct. It will be of great interest in the future to identify the molecular and cellular circuitries underlying ethanol preference using the robust and high throughput assay that we have established in this study.

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Figure 1. Ethanol preference assay in zebrafish

A). Picture showing the conditioned place preference (CPP) chamber. B). Flow-chart of the experimental design.

Figure 2. Initial preference of naïve male AB fish

Initial time spent in the dotted vs. white compartment. Mean percent time $\pm$ SEM values are shown ($n = 38$).

A. Initial preference recording

↓
Conditioned to ethanol
on the dotted side

B. Final preference recording

Figure 3. Typical tracks of zebrafish generated by Ethovision before and after conditioning to ethanol

In this example, the animal spent a greater percent of time on the white side during initial preference recording (A). After conditioning to 1.5% ethanol on the dotted side, the animal spent a greater percent of time on the dotted side as seen in the final preference recording (B). Only verified tracks are used by Ethovision to calculate the distance moved and velocity of the fish.

Figure 4. Ethanol preference behavior measured in the CPP chamber

A) Percent time spent in conditioning compartment before (white bar) and after (black bar) conditioning with 0% (n=8), 0.5% (n=11), 1% (n=11) and 1.5% (n=8) ethanol. Student's t test showed a significant effect of 1.5% ethanol ($p=0.0268$) for before 1.5% ethanol compared with after 1.5% ethanol. B) Changes in preference for the conditioned compartment of control (n=8) and experimental animals treated with 0.5% (n=11), 1% (n=11) and 1.5% (n=8) ethanol. Change in preference = (% time in the ethanol-paired compartment after conditioning) – (% time in the same compartment before conditioning). Mean $\pm$ SEM are shown. Student's t test showed that 1.5% ethanol had a significant effect on change in place preference. (Student's t test, $p=0.0409$ for 1.5% ethanol compared with

0% ethanol). C) Changes in preference for the conditioned compartment of control and experimental animals. Each experimental group (0.5%, 1% and 1.5%) has been divided into two sub-groups depending on whether the animals received ethanol in the first or second conditioning session. Student's t test showed that there was no significant effect of conditioning order on change in preference [$p=0.0956$ for 0.5% EtOH 1st (n=5) vs 0.5% EtOH 2nd (n=6); $p=0.4353$ for 1% EtOH 1st (n=4) vs 1% EtOH 2nd (n=7); $p=0.2043$ for 1.5% EtOH 1st (n=4) vs 1.5% EtOH 2nd (n=4)]. The control and 1.5% EtOH 2nd groups are significantly different ($p=0.0159$).

Figure 5.

Average swimming speed (cm/sec) of zebrafish after 19 min exposure to either 0% (n=24), 0.5% (n=7), 1% (n=11) or 1.5% (n=5) ethanol concentration. No significant effect of 19 min ethanol exposure on swimming speed was detected.

Table 1

Brain ethanol level [mM and % (v/v)] were measured in adult AB zebrafish upon acute treatment with 1.5% ethanol (v/v) in the tank water for 20 minutes.

Treatment	AB	
	mM	% (v/v)
Control	0	0
1.5% Ethanol (v/v) for 20 min, #1	2.35	0.015
#2	1.70	0.011
#3	2.85	0.018
#4	2.16	0.014
#5	2.07	0.013
Average	24.49 $\pm$ 2.07	0.014 $\pm$ 0.001