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Received: 20 February 2023 | Revised: 10 January 2024 | Accepted: 2 March 2024

DOI: 10.1002/bmb.21829

PROTOCOL

Biochemistry and Molecular Biology Education

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Exploring in the classroom the relationship between alcohol intake and behavioral disorders through an animal model

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Funding information
Ministerio de Ciencia e Innovación, Grant/Award Number: PID2021-122896NB-I00; Universidad de Murcia; Fundación Séneca, Grant/Award Number: 21587/FPI/21

Abstract

Alcohol consumption has profound effects on behavior, such as impaired judgment, addiction or even death. It is estimated that alcohol contributes to around three million deaths worldwide, 13.5% of them in young people with ages between 20 and 39 years. Consequently, it is necessary to raise awareness among college and high school students of the risk related to alcohol drinking. The small nematode *Caenorhabditis elegans* is an animal widely used as a model organism to study nearly all aspects of Biochemistry. It is a powerful tool to test the potential bioactivity and molecular mechanisms of natural compounds and drugs *in vivo*. Therefore, it is an interesting topic to include in an undergraduate course of Biotechnology, Biochemistry or Biology students among other scientific vocations. *C. elegans* is also used as a neurobiological model to evaluate substances´ neurotoxicity and behavioral effects. The proposed experiment introduces students to the handling of this preclinical model and to the evaluation of behavioral alterations induced by chemicals in scientific research. The effects of different doses of ethanol on *C. elegans* behavior are studied using a versatile chemotaxis assay. This laboratory experiment is suitable for an undergraduate course. The practical session can be used in the global strategies of information and awareness of educational centres to mitigate the impact of alcohol abuse among students, both in formal courses or in Science fairs or exhibitions.

KEYWORDS

alcohol abuse, biotechnology, biotechnology, consumer chemistry, drugs/pharmaceuticals, hands-on learning, laboratory instruction, scientific practice, toxicology

Samanta Hernández-García and M. Alejandra Guerrero-Rubio are co-first authors.

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1 | INTRODUCTION

Ethanol is a widely consumed substance in diverse cultural contexts with significant behavioral effects such as lack of coordination, impaired judgment, or decreased locomotion. Furthermore, excessive ethanol consumption causes addiction and increases the risk of injury and even death.[1] Alcohol misuse is the third-leading preventable cause of death, and approximately 95,000 people in the United States pass away from alcohol-related causes annually.[2] NIAAA (National Institute on Alcohol Abuse and Alcoholism) statistics estimate that 1519 college students with ages from 18 to 24 die from alcohol-related unintentional injuries, including motor vehicle crashes. Thus, underage alcohol misuse is a global burden that parents, teachers, and college personnel must address.[3] Raising awareness of the harmful effects of consuming alcoholic drinks could be a potential intervention for this problem.

Several studies have reported that the intoxicating effects of ethanol are similar between mammals and invertebrates, suggesting a conserved mechanism of action and also the existence of conserved targets in the nervous system.[4,5] *Caenorhabditis elegans* is a small free-living nematode, widely used as model animal in experimentation.[6] In fact, the Web of Science database shows that 36,710 research papers using this nematode have been published in the last 20 years (Figure 1). *C. elegans* testing provides useful data from a whole animal with metabolically active digestive, sensory and neuromuscular systems.[7] The possible substances to be tested in the model animal range from natural compounds[8,9] to potential synthetic drugs,[10,11] including toxicants. The living model allows not only for the verification of the compounds effects but also may provide a deep understanding of the underlying mechanism of action (MOA) at molecular and genetic levels. This adds up to the interest in replacing and reducing mammalian models in research, and makes *C. elegans* an interesting topic to include in an undergraduate course for scientific vocations students in Chemistry, Biochemistry or Biotechnology.

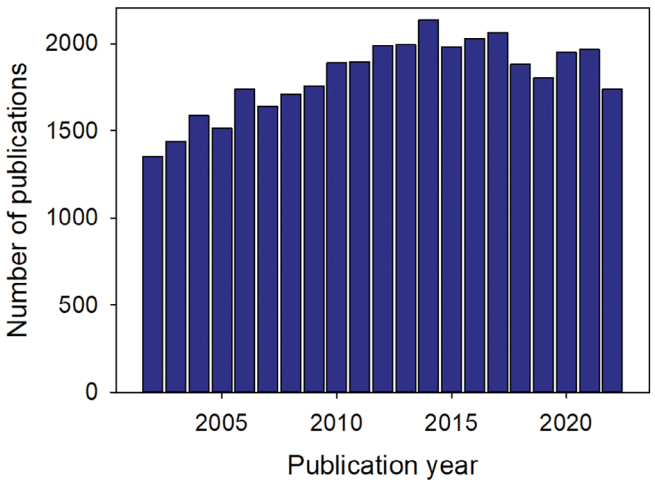

FIGURE 1 *Caenorhabditis elegans* scientific publications obtained from the Web of Science database. Consulted in December 2022 in https://www.webofscience.com/wos/ by searching "Caenorhabditis elegans" on "Topic" and manually filtering publications actually dealing with the nematode as an animal model.

The simple, yet complete nervous system of this animal allows *C. elegans* to be used for studies on the neurobiological basis of behavior in different environments or conditions,[12] including alcohol exposure.[13,14] Experiments with *C. elegans* in this sense may involve the elucidation of molecular basis of the *in vivo* response to the toxic substance[14] or deepen in the analysis of behavioral changes experienced by the exposed organism,[15] including the effect on the speed of movement.[16] In the case of the practical session proposed, a tool to quantify changes in the performance of the nematodes is described and the effect of ethanol exposure is analyzed in terms of the preference and movement towards a defined substance that attracts the nematodes. This is called chemotaxis assay.

The present experiment has been designed to introduce the students to the manipulation and use of the model animal *C. elegans* and to raise awareness of the behavioral effects associated to alcohol consumption. The students expose the small nematodes to different concentrations of ethanol, that is, 0, 5, 10 and 15% v/v, which correspond to the concentrations of ethanol in water, in a regular beer, in table wine and in a cocktail, respectively. Then they subject the treated animals to the chemotaxis assays. The assay assesses the ability of the animals to reach a well-known attractant in specific plates. Neuronal or locomotive-impaired animals fail to reach the attractant and get lost in the plate. In our experience, the attention of students is quickly attracted by the small animals moving on the plates and by the idea of providing them with alcohol. These premises increase the student motivation in the course, their interest in research and provide an exceptional background to discuss the effects of alcohol consumption on behavior and health. The proposed experiment can be performed in one laboratory session of 4 h of an undergraduate course. Students draw and finish the chemotaxis plates, manipulate the nematodes and treat them with the ethanol solutions given to them. The animals are left to move for one hour in the chemotaxis plate, and then the students evaluate their behavior by calculating the chemotaxis index (C.I.). The obtained results are discussed globally in the same session. The experiment has been successfully introduced in a preclinical model for new drug discovery and toxicology course with positive feedback from students.

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2 | EXPERIMENTAL SECTION

2.1 | *C. elegans* maintenance

The *C. elegans* wild-type N2 strain and the *Escherichia coli* OP50 strain were kindly provided by the Caenorhabditis Genetic Centre (CGC, St Paul, MN, USA), funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). The strain maintenance and synchronization were performed following Stiernagle procedures.[17]

2.2 | Reagents

All reagents used in this work were purchased from Fisher Scientific (Massachusetts, USA). *C. elegans* maintenance and chemotaxis plates contained solid nematode growth medium (NGM) (3 g L^{-1} NaCl, 17 g L^{-1} agar, 2.5 g L^{-1} peptone, 5 mg L^{-1} cholesterol, 1 mM $MgSO_4$ in 25 mM potassium phosphate buffer pH 6.0). OP50 was used as a food source, overnight cultures were grown in LB (Luria–Bertani) medium at 37°C and were concentrated 10× in sterile M9 buffer (3 g L^{-1} KH_2PO_4, 6 g L^{-1} Na_2HPO_4, 5 g L^{-1} NaCl, 1 mM $MgSO_4$ pH 6.0).

2.3 | Experimental procedures

2.3.1 | Ethanol treatment

Age synchronized L4 larvae grown in NGM plates were transferred to 1.5 mL tubes and washed with M9 buffer twice. The supernatant was removed, and the worms were treated with 1 mL of an ethanol solution (0, 5, 10 or 15%) for 15 min at 25°C. Then the solutions were removed, and the worms were washed twice with 1 mL of M9. Afterwards the treated worms were used for the chemotaxis assay.

2.3.2 | Chemotaxis assay

The chemotaxis assay was prepared following Margie, Palmer and Chin-Sang's protocol.[18] Briefly, the chemotaxis plates were 55 mm NGM plates, which were divided in 4 equal quadrants, and in the center, a 0.5 cm radius circle was drawn. Each quadrant was marked with either a "T" for "Test" or a "C" for "Control" ensuring that the positions were equidistant from each other and at least 2 cm away from the center. The top left and bottom right quadrants were test quadrants, and the top right and bottom left quadrants were controls. 2 μL of 0.5 M sodium azide and either 10% isoamyl alcohol (dissolved in ethanol) for test quadrants as the attractant or ethanol for control quadrants was added in each mark. Then 10 μL of treated worms were placed in the center of each plate, and the plates were left in the dark at 25°C. The whole experiment is summarized in Figure 2. This procedure was performed in triplicate for each condition. After one hour at 25°C, the animals in each quadrat were counted, and the chemotaxis index was calculated using the Equation (1).

$$C.I = \frac{total\ number\ of\ worms\ in\ T - total\ number\ of\ worms\ in\ C}{total\ number\ of\ worms\ in\ T + C} \quad (1)$$

2.3.3 | Handling of worms with a platinum wire

During the 60 min needed to fully develop the chemotaxis assays, students are trained in the handling of nematodes and, for this purpose, in the manufacture of their own platinum tool (worm picker). Worm picking is widely used in *C. elegans* procedures such as quantifying animals, phenotype scoring, manual assays or crossing/mating strains. Students are provided with a 150 mm glass Pasteur pipette, a piece of platinum wire and tweezers. They insert the wire into the glass pipette and the instructor lights the Bunsen burners. Then the students heat the pipette tip and seal the platinum wire by clamping the hot and malleable pipette tip with the tweezers.[17] Once the wire is secured the students flatten it with the tweezers and use their worm pickers to transfer nematodes from a crowded plate to a clean one. Generally, the wire is scraped against the *E. coli* seeded on the clean plate to make it sticky. Then the picker is placed on a worm, the nematode will stick to the *E. coli*, allowing it to be moved to a clean plate and be released by pressing gently the picker onto the agar surface. The students transfer four or five nematodes and visualize them crawling on the new plate.

2.4 | Hazards

Sodium azide is toxic if ingested or inhaled. Ethanol and isoamyl alcohol are flammable, toxic if ingested or inhaled and irritant in contact with the skin. Safety gloves should be worn during the procedures and changed for clean ones once the azide has been manipulated. Bunsen burner presents fire hazards.

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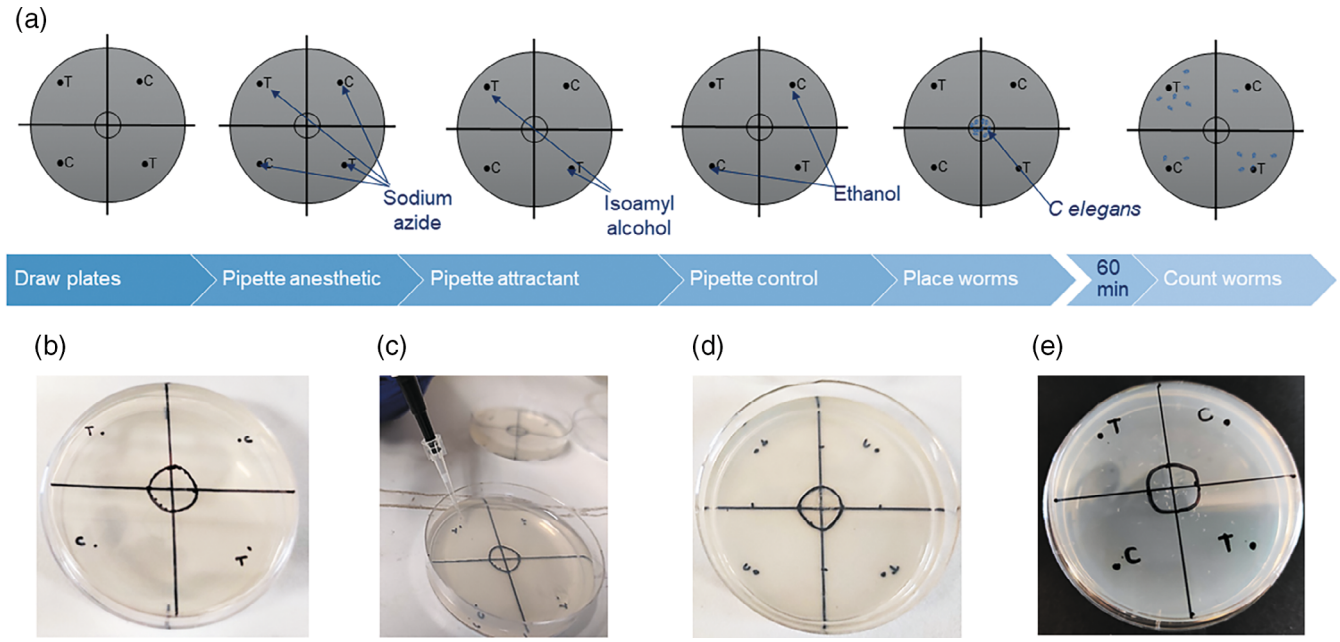

FIGURE 2 Experiment overview. (a) Chemotaxis assay scheme. (b–e) Representative images of a marked chemotaxis plate (b), a plate in preparation (c), a plate just after *C. elegans* are placed on it (d), and a plate one hour after the addition of the animals (e).

2.5 | Ethical considerations

Invertebrate animals such as *Caenorhabditis elegans* are not included in the Directive 2010/63/EU of the European Parliament and of the European Council of 22nd September 2010 "on the protection of animals used for scientific purposes", nor in the Spanish Law 32/2007 of 7th November "for the care of animals, in their exploitation, transport, experimentation and euthanasia" (BOE of 8th November 2007) and Spanish Royal Decree 53/2013 of 1st February 2013 laying down the "basic normative applicable for the protection of animals used in experimentation and other scientific purposes, including teaching" (BOE of 8th February 2013). Furthermore, the US Animal Welfare Act (AWA) currently only applies to warm-blooded animals.[19] Therefore, this laboratory practice does not present any legal implication. However, *C. elegans* is a complete animal and the need of respect and care to avoid any unnecessary disturbance of the specimens is explicitly transmitted to the students, both in the practical session and in the previous classroom sessions. The number of specimens in the practical session is also kept to the minimum. With respect to the students' formal assessment, the Institutional Research Ethics Committee received the procedures completed and concluded that these procedures do not have ethical implications.

3 | RESULTS AND DISCUSSION

The students exposed *C. elegans* young adults to different ethanol concentrations (0, 5, 10 and 15% v/v) for fifteen minutes. Then they placed the exposed nematodes on prepared chemotaxis plates, which are divided into quadrants, where previously sodium azide (anesthetic), isoamyl alcohol (attractant) or ethanol (control) were pipetted (Figure 2). The animals are free to roam in the plate for one hour. However, when they approach to the designated mark (T or C), their movement is slowed down due to the sodium azide placed at the marks. Each student has one ethanol condition and three chemotaxis plates. All the information needed to reproduce the experiment is described in the Data S1, including Reagents, Students' handout, and Instructors´ notes. The obtained results for a 18 student's classroom are shown in Figure 3 and in Table S1. As expected, ethanol exposure impaired the chemotactic ability of healthy *C. elegans* young adults in a dose-dependent manner. Control animals (treated with water) had a chemotaxis index of 0.43, which is within the normal values for wild-type nematodes, and approached the attractant normally. On the other hand, most of the animals treated with ethanol 15% did not move from the plate center. The few animals that moved failed to reach the T quadrants resulting in a lower chemotaxis index of 0.038, a value that can be compared to that obtained by

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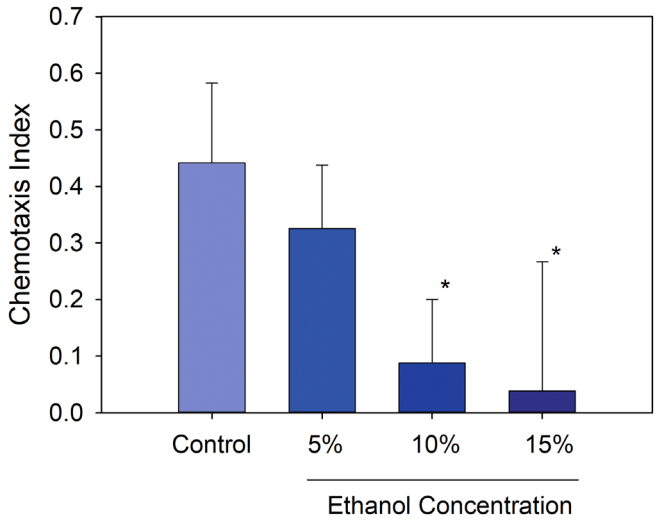

FIGURE 3 Average chemotaxis index obtained for each condition, data is represented as mean ± S.D, * significant at $p \leq 0.05$ by ANOVA test.

transgenic *C. elegans* affected by Alzheimer's disease.[20] Exposure to a 10% ethanol dose also reduced significantly the C.I. (0.08) and worms were unable to reach the attractant properly. Although, the mildest condition (5%) did not significantly reduce the C. I., the values obtained were lower than those for the control, an indication of a mild negative effect of the substance.

The chemotaxis protocol described was able to show the negative effect of ethanol intake by the model animals. The procedure is general and allows for the evaluation of substances that may be harmful to the organisms, or it can be used to evaluate the protecting effect of other molecules or extracts. This protocol is quantitative, shows dose dependence results, and introduces the students to the relevance of statistical analysis and controls on behavioral experiments.

The students were questioned about the conclusion of the experiments, and they realized by themselves that ethanol exposure impaired the animal's perception and locomotion. Some of them verbalized that the same happens to humans when exposed to alcohol. One student also proposed prolonging the experiment time and studying whether the nematodes were able to recover from the ethanol exposure. Furthermore, a formal assessment with a fully validated 14-question inventory on experimental design in Biology (BEDCI) was used to evaluate our students thinking in experimental design[21] ($n = 18$). This was done after the practical session with a reliable tool able to measure non–expert like student thinking regarding experimental design, used here as a mean of assessing the effectiveness of the teaching strategy in improving student learning. Our students (final year, undergraduate course in Biotechnology) performed very well, and their results indicate a good understanding of the principles in the design of experiments in Biology.[21] They only had a weaker response to BEDCI questions 7, 8, and 12, although succeeded in question 13, all related to random-independent sampling. They highly succeeded in stablishing appropriate expert-like opinions on controls (questions 1 and 5) and hypotheses (questions 2 and 9), identifying extraneous factors affecting experiments (question 6 and 14) or accuracy (question 4), and on the relevance of biological variations (question 3 and 10). Although it is difficult to identify a single factor responsible for the good performance of the students, the BEDCI test stablishes a validated reference background from where we can conclude that hand-on learning and experimental design approaches have a positive effect on students. Information and awareness on the effects of ethanol consumption further enrich the learning experience. All needed materials to perform the BEDCI test can be found from the original publication.[21] In this sense, our only modification was the translation to Spanish of the background information and the slides with the questions.

4 | CONCLUSIONS

This experiment was designed for an undergraduate preclinical model for new drug discovery and toxicology course to show the students the applications of *C. elegans* as model animal for research and to introduce them to the handling of nematodes. The experiment highlights the relevance of a quantitative chemotaxis assay to observe behavioral changes in the living organism. Additionally, the laboratory session allows for discussion on scientific research and on the adverse effects of alcohol consumption. The use of living animals that move and reacts to stimuli increases the students' interest in the course. Furthermore, the use of ethanol raises awareness on healthy lifestyle and on the harmful effects of alcohol misuse.

AUTHOR CONTRIBUTIONS

Conceptualization: Samanta Hernández-García and Fernando Gandía-Herrero; **Methodology** and **Formal Analysis**: Samanta Hernández-García, M. Alejandra Guerrero-Rubio, Paula Henarejos-Escudero, Pedro Martínez-Rodríguez and Fernando Gandía-Herrero; **Writing—Original Draft**: Samanta Hernández-García, Paula Henarejos-Escudero, and Pedro Martínez-Rodríguez; **Writing—Review and Editing**: Fernando Gandía-Herrero; **Investigation**: Samanta Hernández-García, M. Alejandra Guerrero-Rubio, Paula Henarejos-Escudero, Pedro Martínez-Rodríguez; **Supervision**, **Project Administration and Funding Acquisition**: Fernando Gandía-Herrero. All authors have read and agreed to the published version of the manuscript.

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ACKNOWLEDGMENTS

This work was supported by the Spanish Ministry of Science and Innovation, project PID2021-122896NB-I00 (MCI/AEI/1013039/501100011033/FEDER, UE). P. M.-R. holds a contract financed by Fundación Séneca (Spain) (21587/FPI/21). P. H.-E. holds a contract financed by University of Murcia (Spain).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Hernández-García S, Guerrero-Rubio MA, Henarejos-Escudero P, Martínez-Rodríguez P, Gandía-Herrero F. Exploring in the classroom the relationship between alcohol intake and behavioral disorders through an animal model. Biochem Mol Biol Educ. 2024;52(4):474–9. https://doi.org/10.1002/bmb.21829

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