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Effects of Nicotine on the Cyp6a8 gene promoter of Drosophila melanogaster

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ABSTRACT

Cytochrome P450 monooxygenases (CYPs) are enzymes that are found in all living organisms, and they serve a protective function by metabolizing toxic agents that enter the body. In previous studies, it has been found that caffeine induces the transcriptional activity of the Cyp6a8 promoter. The present study focuses on the transcriptional effects of another xenobiotic as well as toxic chemical – Nicotine. To measure this, transgenic Drosophila strain carrying the firefly *luciferase* reporter gene under the control of 0.8-kb promoter DNA of the Cyp6a8 gene were used. Adult female flies of this strain were treated with varying concentrations of Nicotine for 24-48 hours. The treated and control flies were then homogenized and centrifuged to make extracts. Each of these extracts were then assayed for luciferase activity and protein concentration. Results showed that Nicotine significantly induced transcription of the Cyp6a8 promoter. This result is important considering the prevalence of Nicotine use in the world and the adverse effects it has on the human body.

INTRODUCTION

Cytochrome P450s are ubiquitous enzymes with many evolutionarily conserved structures between animal and plant species (Kumar et al., 2014). One purpose these enzymes serve is to modify endogenous substances in the body, such as steroids, bile acids, and vitamins (Guengerich, 1993). Another well-known task of CYPs is to metabolize xenobiotic (foreign) compounds, including seemingly harmless household chemicals, drugs, and potent carcinogens (Guengerich, 1993; Bhaskara et al., 2006). In humans, a person's response to a drug depends partly on the active P450 enzymes contained in that person's body; if they lack an adequate amount of CYP activity, they are unable to eliminate the drug from their system, which leads to its continual accumulation (Guengerich, 1993). In plants, a study on antipredator herbivore defense showed that CYP6B46 was required to convert nicotine into a transportable metabolite for its efflux from larval midgut to hemolymph (Kumar et al., 2014). CYP genes have known involvement in conferring insecticide resistance to pesticides, and many insect species have displayed insecticide resistance-associated overexpression of some CYP genes (Bhaskara et al., 2006). For example, In Drosophila, overexpression of at least five Cyp genes has been seen in DDT resistant strains (Bhaskara et al., 2006). In these DDT-resistant strains, enhanced detoxification of DDT by cytochrome P450-dependent monooxygenase enzymes is seen (Denholm et al., 2002). Other studies have shown the ability of many common chemical compounds, such as alcohol and cigarette smoke, to induce or suppress activity of these genes (Guengerich, 1993), which points to the opportunity for further research regarding drug use and its effect on gene expression.

Previous studies have examined various chemicals and their effects on the expression of cytochrome P450 enzymes, one of which demonstrated caffeine's ability to induce P450 gene transcription in adult female flies by treating with the caffeine pill Vivarin (Bhaskara et al., 2008). Two other studies also showed increased transcription at the Cyp6a8 promoter in flies treated with caffeinated tea (Scott, 2015; Retzer, 2017). Another substance that is widely used throughout the world is Nicotine. Although it is not used quite as much as caffeine, the prevalence of individuals who eventually become Nicotine-dependent is higher than that of any other substance abuse disorder (Kandel et al., 1997). This indicates the high addictive potential contained within Nicotine, prompting more questions regarding possible consequences of its widespread use. Extensive studies have been done to analyze the

behavioral effects of Nicotine and its addictive properties, but not near as many have explored Nicotine's effects on gene expression. Of the few that have been done, one revealed Nicotine's ability to alter transcription by inducing long-term pCREB in chick ciliary ganglion neurons (Chang et al., 2001). Another study on patients with non-small-cell lung cancer showed differences in nicotinic acetylcholine receptor subunit genes between smokers and non-smokers, with higher levels in non-smokers (Lam et al., 2007). Both of these studies demonstrated Nicotine's ability to alter gene expression in different species. However, studies that examine nicotinic effects on CYP transcription are currently lacking. Therefore, in the present study analyzed Nicotine's effects on the Cyp6a8 gene promoter in adult female flies. The results revealed a significant level of gene induction in flies treated with 0.1% Nicotine compared to the control flies treated with water.

MATERIALS AND METHODS

1. *Fly strains and culture conditions*:

A reporter transgenic line of *Drosophila melanogaster* called 0.8-luc110H-ry (Maitra et al., 2002) was used to determine nicotine's effects on transcription at the *Cyp6a8* gene promoter. This transgenic strain is homozygous for a single copy of 0.8-luc reporter transgene, which is located on chromosome 2 of the ry^{506} host strain. This luciferase reporter gene is under the control of a 0.8 (-11/-761) kb upstream DNA of the Cyp6a8 gene (Maitra et al., 2002; Bhaskara et al., 2008). To minimize possible sex-dependent variation, only female flies were used in the study.

2. *Preparation of Nicotine solution*:

Nicotinic Acid was purchased from Sigma (St. Louis, MO) in a concentration of 40%. To determine the proper concentration to treat flies with, toxicities of various concentrations were examined by treating flies of the 91R (DDT-resistant) and 91C (susceptible) strains and noting survival rates after 24 hours. After trial experiments using 1% and 0.1% Nicotine solutions, 0.1% was chosen as the ideal dosage for the experiment due to the high number of deaths caused by the 1% solution within 24 hours. The 40% stock solution was then diluted to a concentration of 0.1% by mixing with sterile water.

Table 2.1. DDT-resistant (91R) and susceptible (91C) strains were treated with varying concentrations of Nicotine to examine the toxicity of each.

Time Elapsed	1.0% Nicotine		Water		0.1% Nicotine		Water	
	91C	*91R*	*91C*	*91R*	*91C*	*91R*	*91C*	*91R*
24 hours	14/19 dead	1/19 dead	0/19 dead	0/19 dead	0/19 dead	0/19 dead	0/19 dead	0/19 dead
48 hours	19/19 dead	3/19 dead	2/19 dead	1/19 dead	1/19 dead	0/19 dead	0/19 dead	1/19 dead

3. Treatment of transgenic flies:

Instant fly food blue color indicator (Carolina Biologicals) was used in both the treatment and control vials, the former saturated with 0.1% Nicotinic Acid and the latter with sterile water. In each glass vial, 6 grams of fly food were saturated with 2 mL of liquid to properly moisten the food. Flies were then etherized and 15 females sorted into the Nicotine vial and 15 females into the control vial. Cotton plugs were then pushed down to the halfway mark on the vial to encourage flies to eat more. This "force-feeding method" ensured the flies ingested adequate amounts of either Nicotine or water. Once the flies were awake, they were placed in a dark cabinet for 48 hours of treatment.

4. Preparation of fly extracts:

After undergoing treatment for 48 hours, the flies were etherized again and sorted into groups of 3. The most plump, healthy-looking flies with blue bellies were used. Groups of three flies were sorted into individual 1.5mL Eppendorf tubes, and three individual groups were used for both the control and the treated flies. After the flies woke up again, they were placed on ice to knock them down and 150 μL of 1X Cell Culture Lysis Reagent (CCLR, Promega) was added to each tube. Immediately after adding CCLR, a handheld power homogenizer was used to thoroughly homogenize the flies. The homogenates were then centrifuged at 13,000 RPM for 8 minutes at room temperature to separate the supernatant from the lipid layer and other cell debris. Supernatant (100 μL) was carefully removed and transferred to a new 1.5 mL Eppendorf tube for each of the six tubes. They were centrifuged again at 13,000 RPM for 8 minutes at room temperature, and then 50 μL supernatant was removed and transferred to 0.5 mL Eppendorf tubes. These final extracts were stored at -20°C until used for luciferase and protein assays.

5. Luciferase assay:

The entire operation was performed in a room under dim lighting using a Luciferase assay reagent (Promega) and a luminometer machine (Zylux). Extracts were taken from the -20°C conditions, allowed to thaw, and placed on ice. LAR (luciferase assay reagent) was taken out of -80°C freezer placed on ice to thaw. To measure luc activity, 25 μL LAR was placed at the bottom of a 1.5 mL Eppendorf tube, followed by the addition of 5 μL of cell extract. The tube was quickly closed, gently mixed with 5-6 taps, and placed into the luminometer for 15 seconds. The light intensity displayed as RLU/sec (relative light units per second) by the luminometer was recorded for all samples.

6. Protein assay:

This assay was completed using a BCA protein assay kit (Pierce) to determine how much protein was present in each extract. A 1 mg/mL stock solution of Bovine Serine Albumin (BSA) made in 0.5 X CCLR buffer was used to generate a protein standard curve. BSA was added to duplicate test tubes in amounts of 0, 12.5, 25, 37.5, and 50 μg prior to the addition of 0.5X CCLR in various values to make the total amount of liquid per tube equal to 50 μL for all standards. For example, a tube that contained 37.5 μL BSA would have 12.5 μL of 0.5X CCLR added, making the sum of both substances 50 μL. To each of these 10 tubes, 1 mL of BCA Reagent (prepared by mixing BCA Protein Assay Reagent A with BCA Protein Assay Reagent B in a ratio of 50:1) was added. In addition to these 10 duplicate tubes, 2 more were used that did not serve as protein standards. Similarly, each fly extract was assayed in duplicate test tubes. To do this, in each test tube 45 μL 0.5X CCLR and 5 μL fly extract were mixed and 1 mL BCA Reagent was added. All tubes were then placed in a 37°C incubator for 30 minutes to develop their color. Following the 30 minute incubation, each tube was placed in a spectrophotometer (BioMate 3) to measure absorbance at a wavelength of 595 nm. Once all values were obtained, the mean value of protein content (μg) per μL extract was calculated for each set of duplicates. These values were then applied to the luciferase assay data to determine the RLU/μg protein.

7. Statistical analysis:

The absence or presence of statistical significance was determined by calculating T-tests and standard deviations for each trial in Excel. These values are displayed in the tables below.

RESULTS AND DISCUSSION

To determine the effect of Nicotine on the activity of the Cyp6a8 gene promoter, female flies from the 0.8luc110H-ry strain were treated with 0.1% Nicotine for 48 hours. In trial 1, the data shows induction of the promoter in flies treated with Nicotine compared to those treated with water (i.e. the control), and this induction is significant (p-value: 0.029). This is made evident by the greater RLU values in Table 1 for flies treated with Nicotine, indicating increased luciferase gene expression. The average fold induction for flies treated with 0.1% Nicotine is 2.880, which is shown in table 1 and figure 1 below.

Table 1. Effects of 0.1% Nicotine on the transcriptional activity of Cyp6a8 promoter (Trial 1).

Sample	Treatment	RLU/5uL extract	ug protein /5uL extract	RLU/ug protein	Mean RLU/ug protein	SD	Fold Induction	P-value
1	0.1% Nicotine	1,508,538	2.690	560,794.80				
2	0.1% Nicotine	1,134,823	3.211	353,472.36	437,134.84	190,295.46		
3	0.1% Nicotine	2,041,286	5.140	397,137.35			2.880	0.029
4	H_2O	998,258	3.762	265,353.00				*
5	H_2O	332,053	4.249	78,148.51	151,783.25	99,787.20		
6	H_2O	582,282	5.206	111,848.25				

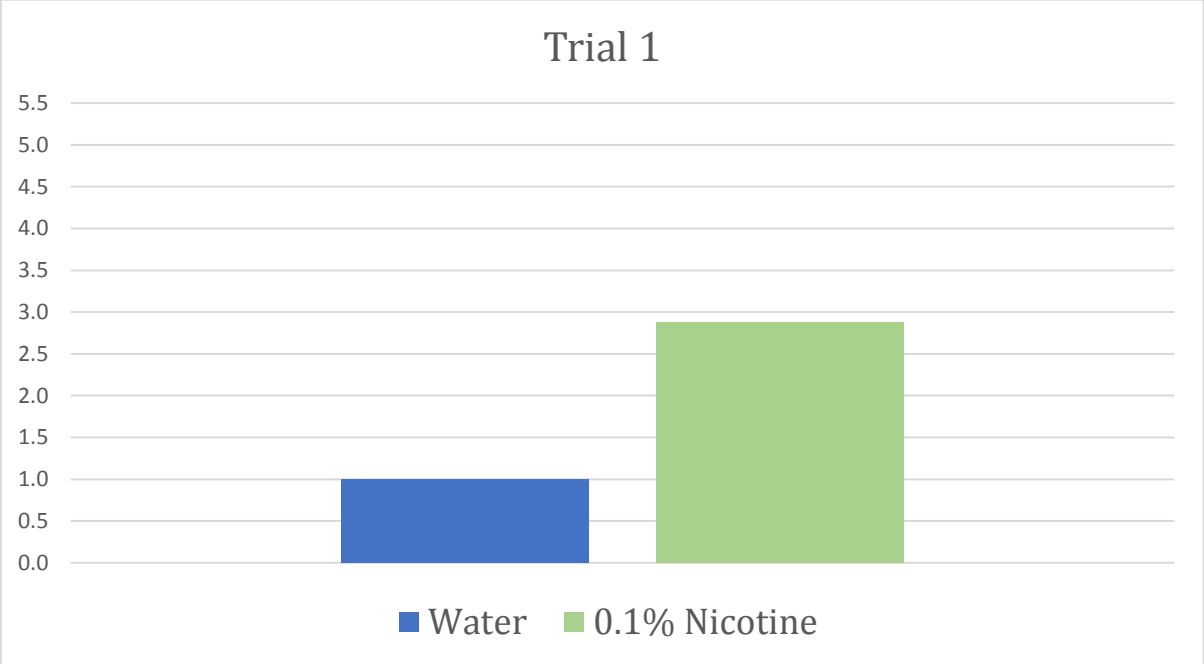

Figure 1. Induction of transcription at the Cyp6a8 gene promoter in female flies treated with 0.1% Nicotine solution (Trial 1).

The data from trial 2 also depicts a strong trend of induction at the Cyp6a8 promoter of flies treated with Nicotine, even though it is not statistically significant (p-value: 0.134). Fold induction of nicotine-treated flies was 3.095. The values in red from sample 5 were outliers and were not included in the mean calculation. These outliers could have been due to human error during homogenization of the extract making process.

Table 2. Effects of 0.1% Nicotine on the transcriptional activity of Cyp6a8 promoter (Trial 2).

Sample	Treatment	RLU/5uL extract	ug protein /5uL extract	RLU/ug protein	Mean RLU/ug protein	SD	Fold Induction	P-value
1	0.1% Nicotine	2,084,566	2.647	787,520.21				
2	0.1% Nicotine	1,538,063	5.235	293,803.82	564,290.26	250,227.60		
3	0.1% Nicotine	1,432,854	2.343	611,546.73			3.095	0.134
4	H_2O	506,511	3.043	166,451.20				
5	H_2O	*1,315,683*	*3.145*	*418,341.18*	182,342.83	22,474.16		
6	H_2O	647,632	3.267	198,234.47				

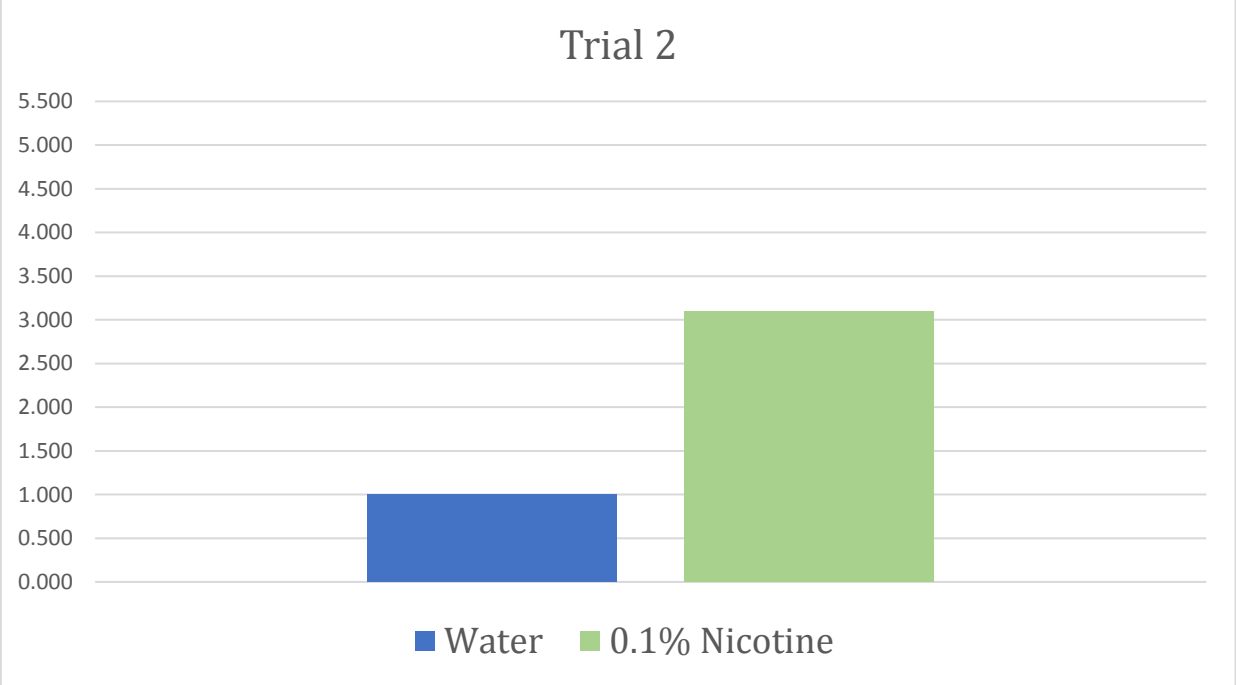

Figure 2. Induction of transcription at the Cyp6a8 gene promoter in female flies treated with 0.1% Nicotine solution (Trial 2).

Trial 3 also contained outliers not calculated in the mean value, which were determined to be sample 3 and 4. These values could have been skewed due to premature death of flies that occurred before ingesting adequate Nicotine, or it could have been due to human error during extract synthesis as well. The data from this trial was not statistically significant either, with the T-test yielding a p- value of 0.108. The same trend is still seen, however, because the Nicotine-treated flies show a much higher RLU and the average fold induction is 5.036.

Table 3. Effects of 0.1% Nicotine on the transcriptional activity of Cyp6a8 promoter (Trial 3).

Sample	Treatment	RLU/5uL extract	ug protein /5uL extract	RLU/ug protein	Mean RLU/ug protein	SD	Fold Induction	P-value
1	0.1% Nicotine	1,346,489	1.186	1,135,319.56				
2	0.1% Nicotine	3,386,880	1.654	2,047,690.45	1,591,505.0	645,143.64		
3	0.1% Nicotine	*861,264*	*2.040*	*422,188.24*			5.036	0.108
4	H_2O	*40,131*	*1.360*	*29,508.09*				
5	H_2O	403,149	1.324	304,493.20	316,039.62	16,039.62		
6	H_2O	656,810	2.005	327,586.03				

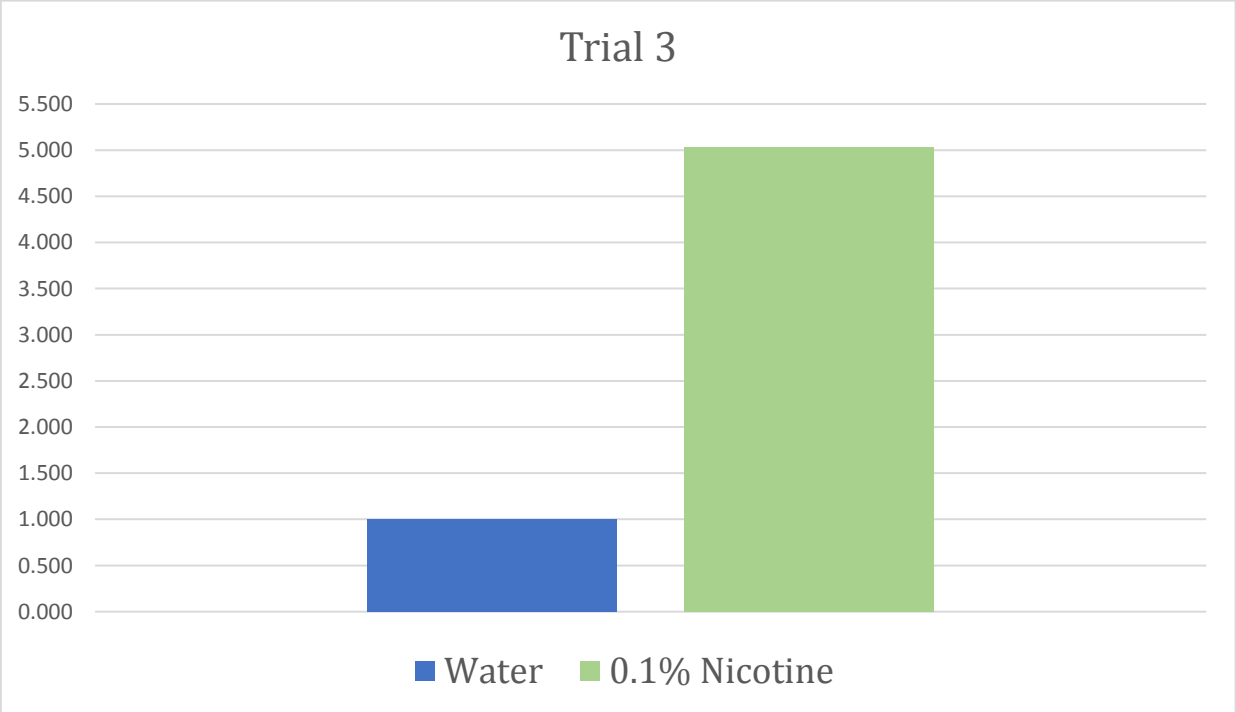

Figure 3. Induction of transcription at the Cyp6a8 gene promoter in female flies treated with 0.1% Nicotine solution (Trial 3).

Data from trial 4 was similar to trail 1 in the absence of outliers and the statistical significance of the induction (p-value: 0.031). The average fold induction of nicotine-treated flies compared to control flies was 2.265.

Table 4. Effects of 0.1% Nicotine on the transcriptional activity of Cyp6a8 promoter (Trial 4).

Sample	Treatment	RLU/5uL extract	ug protein /5uL extract	RLU/ug protein	Mean RLU/ug protein	SD	Fold Induction	P-value
1	0.1% Nicotine	14,909	1.773	8,408.91				
2	0.1% Nicotine	11,020	2.155	5,113.69	6,429.16	1,745.16		
3	0.1% Nicotine	14,441	2.505	5,764.87			2.265	0.031 *
4	H_2O	4,498	1.718	2,618.16				
5	H_2O	3,625	1.628	2,226.66	2,838.14	746.207		
6	H_2O	6,642	1.810	3,669.61				

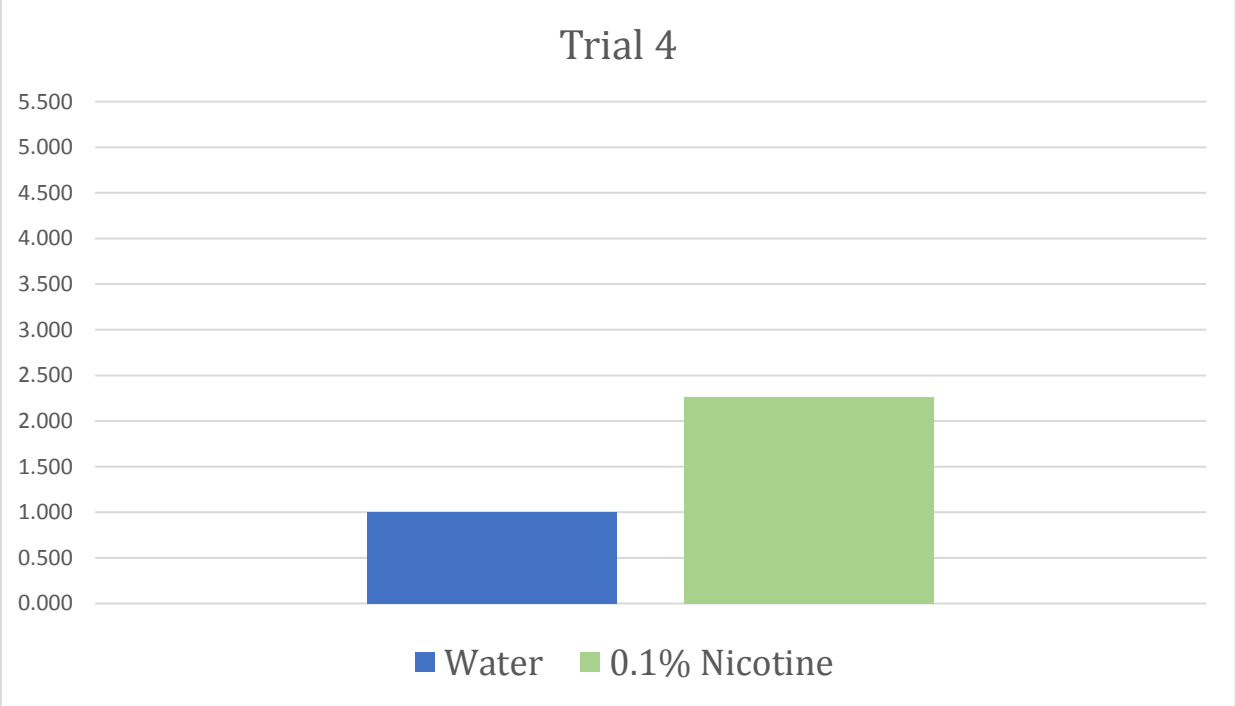

Figure 4. Induction of transcription at the Cyp6a8 gene promoter in female flies treated with 0.1% Nicotine solution (Trial 4).

After analyzing the collected data, the results are consistent throughout all four trials. Treating flies with Nicotine leads to transcriptional induction at the Cyp6a8 gene promoter. This is important considering the growing rate of nicotine use in the U.S. and its possible biochemical consequences. Exploring the effects of overactive CYPs is also of great value considering the ends of many human pathways they take part in, including those involved with cancer. Future studies could further explore nicotine resistance in insects, especially in flies from the 91C and 91R strains. It has been established that they differ in their tolerance and resistance to DDT, but future studies may shed light on each strains' LD50 for Nicotine.

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