

The Effects of Nicotine and Ethanol on the Embryo Development of Zebrafish

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Abstract

The aim of this experiment was to test the effects various concentrations of nicotine and ethanol had on the embryo development of zebrafish. These results could then be used to predict how human embryos would be affected by the same chemicals. This was investigated by exposing zebrafish embryos to varying 30 mM, 100 mM, and 300 mM concentrations of ethanol and 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, and 0.2 mg/mL concentrations of nicotine. Over the course of 72 hours, their growth was monitored and observed. These results show that zebrafish exposed to ethanol and nicotine had a significantly higher death rate and had mutations such as curved spines and pericardial edema. In the highest concentration of ethanol, six fish died and in the highest concentration of nicotine, seven fish died. These are both higher than the 2 fish death seen in the control group. From these results, it can be concluded that nicotine and ethanol are both toxic to zebrafish embryos. Using this data, it can be inferred that these chemicals would also be deadly and cause birth defects in human embryos.

Introduction

The purpose of this experiment was to examine the effects ethanol and nicotine have on embryo development in zebrafish, and use these results to predict how various chemicals would affect humans. From there, mothers could be educated on how ingesting these chemicals during pregnancy could be disastrous, not only to themselves, but to their children as well.

Zebrafish were used in this experiment as a model for human health. This is due to the fact that zebrafish have a high degree of homology with humans (National Centre for the Replacement Refinement and Reduction of Animals in Research, 2014). This means they have similar protein and gene sequences that cause them to react to outside forces in a similar way to how humans would. Furthermore, zebrafish are vertebrates like humans. Mice also have these characteristics and have previously been used for experiments similar to this one. However, it is difficult for scientists to see causes of embryonic lethality in the rodents (Browder & Iten, 1994). This means that because mice are not transparent, it is hard to see mutations that arise within embryos during their development that cause birth defects or death. To do so would require expensive imaging techniques. Therefore, many scientists have begun using zebrafish after

realizing they have many advantages. These include being relatively cheap to maintain, having a small body that is easy to store (between 2.5 and 4 cm), being transparent enough to observe with a low-power microscope, and having a short development time (Browder & Iten, 1994). All of these things make zebrafish an ideal model for human health that can be used to test hypotheses deemed unsuitable for human embryos.

One of the chemicals used in this experiment was ethanol. Ethanol, sometimes called drinking alcohol, is an intoxicating substance found in alcoholic beverages. It enters the bloodstream and circulates throughout the body until it enters the brain and dulls neurological activity (National Institute on Drug Abuse, n.d). A study performed at Western Kentucky University tested the effects high and low concentrations of ethanol had on zebrafish embryos. In higher concentrations, physical deformities and high death rates occurred, while small eye size and low heart rates were observed in lower concentrations (Bilotta, Barnett, Hancock & Saszik, 2004). This shows that in both concentrations, ethanol had a negative effect on zebrafish development. Similarly, the chemical has been linked with embryonic development issues in humans. A study performed by the University of California-Riverside has shown that alcohol consumption during pregnancy can greatly disrupt the neurological development of regions of the brain such as the frontal cortex (University of California Riverside, 2013). This region handles decision-making, social interaction, and learning ability. If a mother drinks while pregnant, a fetus has the chance of developing disabilities such as autism and Fetal Alcohol Syndrome Disorder that can cause learning disabilities, anxiety, lowered intelligence, and behavioral issues like irritability (University of California Riverside, 2013). This supports the idea that ethanol damages a growing fetus.

The other chemical used in this experiment was nicotine. Nicotine is a chemical produced by certain plants, such as tobacco, that absorbs into the leaves and follows the plant when it is processed into various products such as cigarettes (Mandal, 2014). Whether smoked, sniffed, or chewed, nicotine enters the bloodstream and circulates throughout the body. When it reaches the brain it disguises itself as acetylcholine, a neurotransmitter, and disrupts brain activity causing changes in mood, heart rate, and motor skills (Mandal, 2014). An investigation performed by American University, tested the effects of nicotine on the development of zebrafish. These

results show the fish had smaller eye size and shorter notochords, as well as a slower response to stimuli (Parker & Connaughton, 2007). By not being able to react and adapt to their surroundings, many died. These negative effects have also been seen in humans and can lead to an increased risk in premature births. Many mothers that smoke during pregnancy see, on average, a 187 gram decrease in birthweight than that of a child born to a non-smoker (Wickstrom, 2007). In addition, nicotine can damage the brain cells of a child during pregnancy and lead to a higher risk of said child developing ADHD or a drug-dependence later in life (Wickstrom, 2007). In both humans and zebrafish, nicotine can damage embryos and lead to a life of physical and mental complications.

Based on the previous information, I predict that if zebrafish embryos are exposed to ethanol and nicotine then they will have high mortality rates and birth defects because ethanol and nicotine have shown to be toxic to developing fetuses.

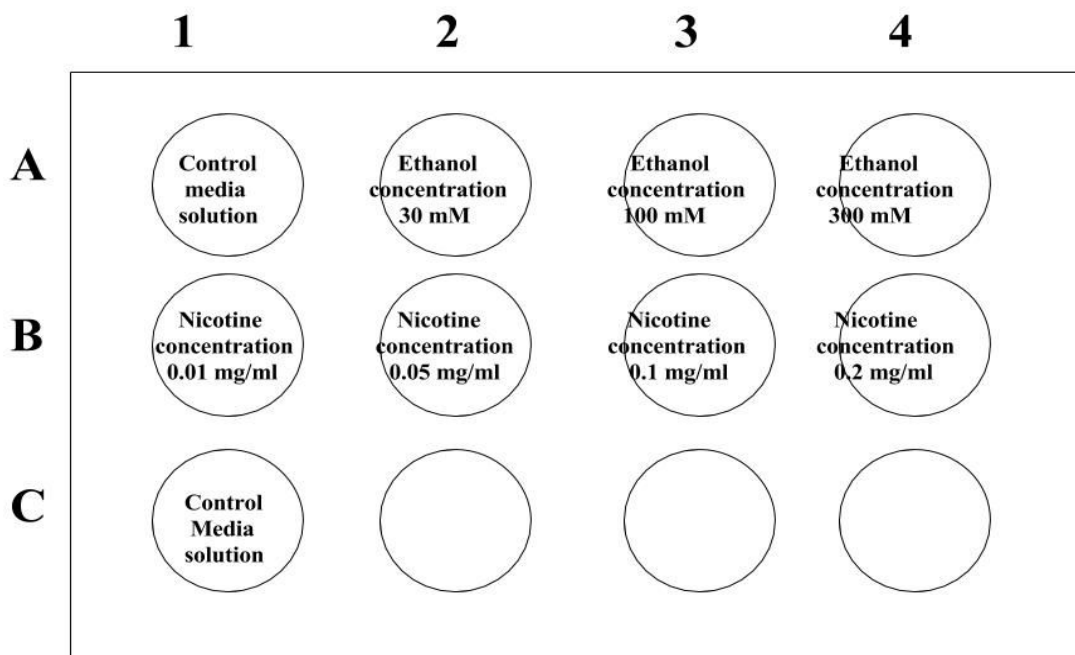
Material List

- 64 Zebrafish embryos
- Bottles of 30 mM, 100 mM, and 300 mM concentrations of ethanol
- Bottle of 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, and 0.2 mg/mL concentrations of nicotine
- Bottle of Embryo Media Solution (control substance)
- 3x4 multi-well plate
- Small and large bore transfer pipettes
- 100 mL beaker for dead embryos and liquid disposal
- Dissecting and Compound microscope
- 28.5°C incubator
- Permanent marker and tape for labeling
- iPhone 5c to take pictures

Methodology

We separated eight live zebrafish embryos into each well of the multiwell plate. Each well was then filled with 2 mL of control media solution or the varying concentrations of ethanol and nicotine shown in Diagram 1. The next day we looked at each well for dead or hatched fish and noticed any changes in the live ones. We wrote down our observations, took pictures of each well, and recorded videos to measure the heart rates of the control well and the wells with the highest concentrations of the two chemicals. Once all of our data collection was over for that day, we used the pipettes to remove dead embryos, debris, and old solution. Then we refilled each well with 2 mL of its corresponding liquid and placed the plate in a 28.5°C incubator. This process was repeated at the 48 hour and 72 hour post fertilization period. Once the experiment was over, we finalized our data and disposed of all fish, debris, and liquid.

Diagram 1

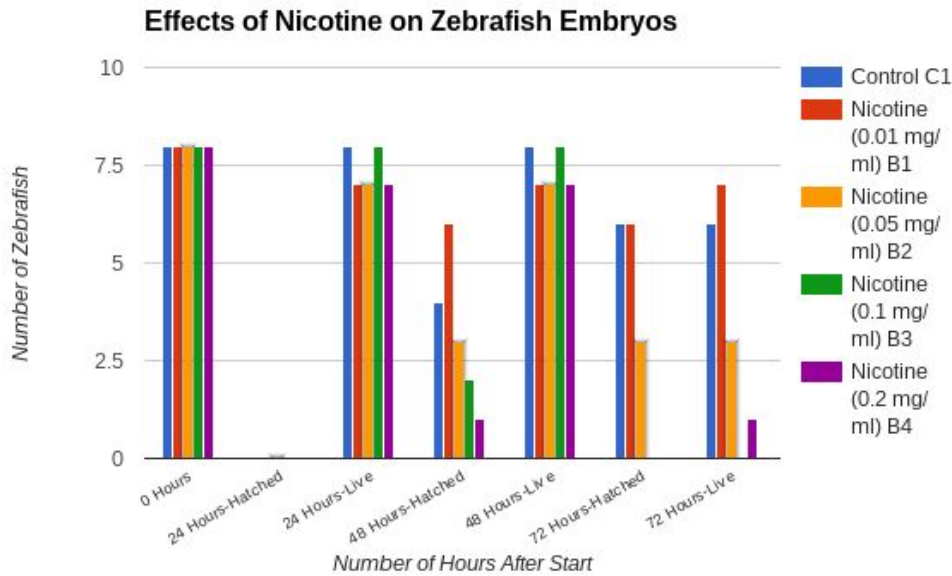


Results

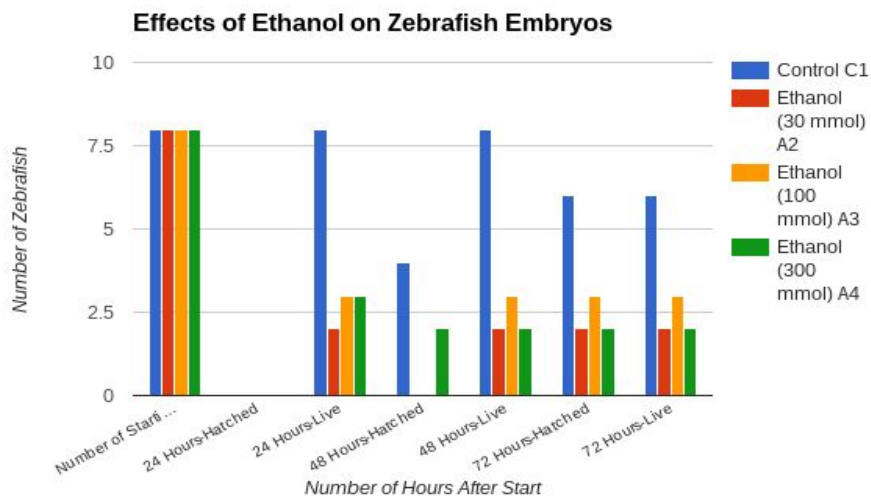
Number of Surviving Zebrafish Embryos Exposed to Ethanol and Nicotine for Three Days

Treatment	Well Number	Start	24 Hours Post Fertilization	24 Hours Post Fertilization	48 Hours Post Fertilization	48 Hours Post Fertilization	72 Hours Post Fertilization	72 Hours Post Fertilization
			# Hatched	# Live	# Hatched	# Live	# Hatched	# Live
Control	C1	8	0	8	4	8	6	6
Nicotine (0.01 mg/mL)	B1	8	0	7	6	7	6	7
Nicotine (0.05 mg/mL)	B2	8	0	7	3	7	3	3
Nicotine (0.1 mg/mL)	B3	8	0	8	2	8	0	0
Nicotine (0.2 mg/mL)	B4	8	0	7	1	7	0	1
Ethanol (30 mM)	A2	8	0	2	0	2	2	2
Ethanol (100 mM)	A3	8	0	3	0	3	3	3
Ethanol (300 mM)	A4	8	0	3	2	2	2	2

Table 1: This table shows the number of living and hatched zebrafish embryos from wells containing 2 mL of varying nicotine and ethanol concentrations as well as media solution that was used for a control. The data was collected over a three day period.



Graph 1: This graph was made using the data from Table 1, focusing on the nicotine side. It shows the number of hatched and living zebrafish that were exposed to varying nicotine concentrations and media control solution for three days (wells C1, B1, B2, B3, B4).

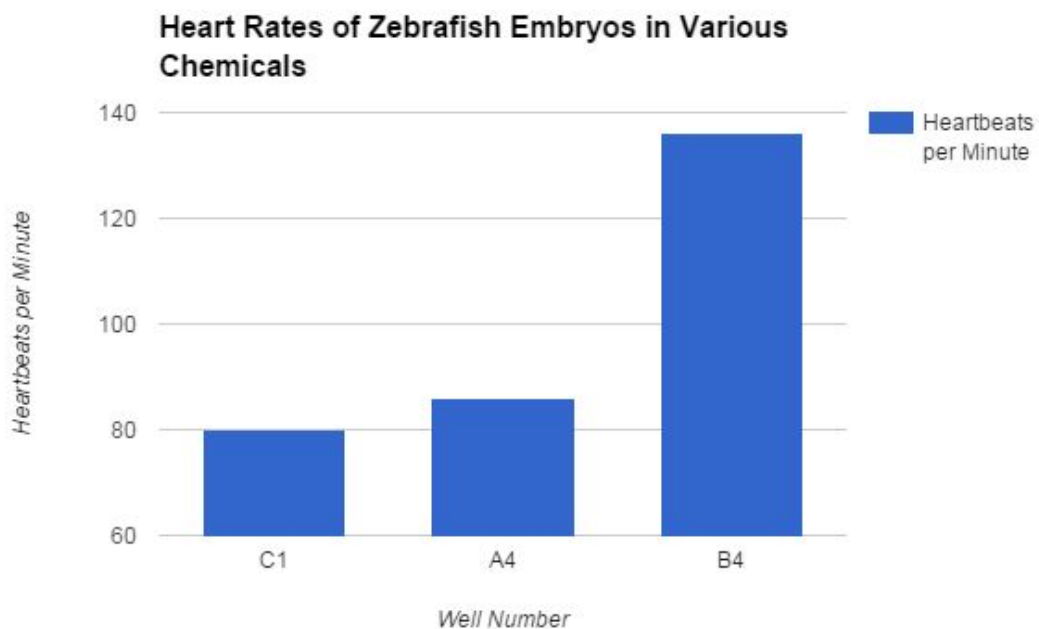


Graph 2: This graph was made using the data from Table 1, focusing on the ethanol side. The graph shows the number of hatched and live zebrafish that were exposed to varying concentrations of ethanol and media solution for three days (wells C1, A2, A3, A4).

Heart Rate of Zebrafish Embryos in the Highest Concentration of Chemicals taken 48 Hours Post Fertilization

Well Number	Treatment	Heartbeats per Minute
C1	Control	80
A4	Ethanol (300 mM)	86
B4	Nicotine (0.2 mg/mL)	136

Table 2: This table shows the heartbeats per minute in the highest concentration of chemicals. Well number C1 was the control group that was exposed to 2 mL of media control solution. Well number A4 was exposed to 2 mL of the highest concentration of ethanol (300 mM), and well number B4 was exposed to 2 mL of the highest concentration of nicotine (0.2 mg/mL). This data was collected 48 hours after fertilization.



Graph 3: (Goes with Table 2) This graph shows the heart rates of a zebrafish in the strongest concentration of ethanol and nicotine, as well as in the media control solution. The well numbers coincide with the above table. A zebrafish in well C1 (the control) had a heart rate of 80 bpm, a zebrafish in well number A4 (300 mM concentration of ethanol) had a heart rate of 86 bpm, and a zebrafish well number B4 (0.2 mg/mL concentration of nicotine) had a heart rate of 136 bpm. The data was gathered 48 hours post fertilization.

Images

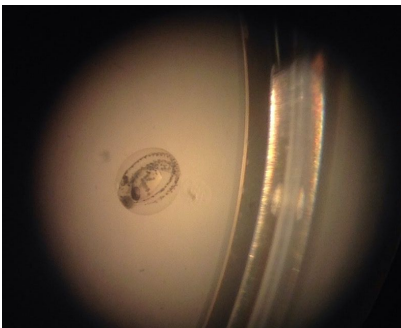


Figure 1

Well: C1

Time: 24 Hours Post Fertilization

Description: This image shows an egg in the control well after 24 hours. The egg looks healthy and has the characteristic black eyes of a zebrafish. In addition, it has a yolk sac that is snug to the body.

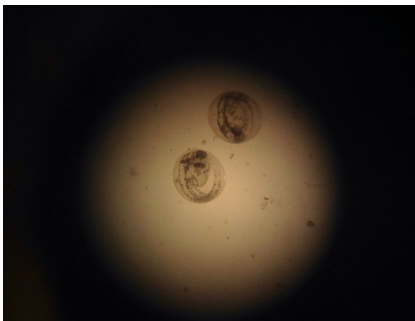


Figure 2

Well: B4

Time: 24 Hours Post Fertilization

Description: This picture shows an egg in the 0.2 mg/mL concentration of nicotine. Although both eggs have healthy shaped eyes and sacs, one shows a malformation. The one further down, seems to have his tail wrapped around its head.



Figure 3

Well: B1

Time: 48 Hours Post Fertilization

Description: This image shows a hatched zebrafish in the 0.01 mg/mL concentration of nicotine. The fish shows signs of pericardial edema, or an accumulation of fluid around the heart cavity.

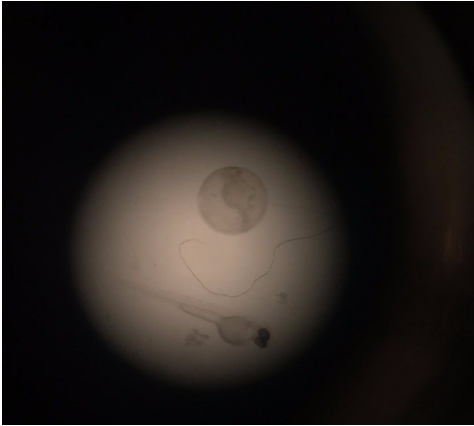


Figure 4

Well: A4

Time: 48 Hours Post Fertilization

Description: This picture shows a dead egg in a 300 mM concentration of ethanol. The egg has a cloudy and mushy look which shows the embryo inside is no longer living.



Figure 5

Well: B3

Time: 48 Hours Post Fertilization

Description: This image shows a hatched zebrafish in a 0.1 mg/mL concentration of nicotine. As shown in the image, the fish does not have the characteristic straight back usually seen in the breed.



Figure 6

Well: A1

Time: 72 Hours Post Fertilization

Description: This picture shows a hatched zebrafish from the 30 mM concentration of ethanol. As can be seen from the image, the fish has an extremely twisted back. This is quite abnormal and does not look like the typical straight spine of a zebrafish.

Results Interpretation

The results of the experiment included the number of alive and hatched zebrafish in each well. In the control well, six zebrafish survived and hatched during the three day observation period. In the ethanol wells, every concentration left only two or three zebrafish alive. The ones that remained all hatched. In the nicotine wells, as the concentration increased, the number of zebrafish that died increased. For example, while only one fish died in the 0.01 mg/mL well (B1), seven fish died in the 0.2 mg/mL well (B4). In addition, data was collected on the heart rate of the fish in the control, 300 mM concentration of ethanol, and the 0.2 mg/mL concentration of nicotine. Both the ethanol fish and the control fish had a heart rate close to 80 bpm. However, the nicotine fish had a heart rate of 136 bpm. Finally, several images were taken to document the development of the zebrafish. In the control group no malformations or birth defects were seen. However, some were seen in the other wells. For example, in well B4 (the 0.2 mg/mL concentration of nicotine), one of the embryos seemed to have its tail twisted around its head while in its egg. Also, in well A2 (the 30 mM concentration of ethanol), a hatched zebrafish had a severely twisted back. Furthermore, in well B1 (the 0.01 mg/mL concentration of nicotine), a hatched fish showed signs of pericardial edema, or an accumulation of fluid around the heart cavity.

Conclusion

After the three day observation period, it was clear that the zebrafish had been affected by the concentration of chemicals that were placed in their well. As expected, the control group had few abnormalities and a high survival rate. For example, of the eight starting fish, six hatched and survived past the 72 hour mark. In addition, as shown in Figure 1, the media solution used in the control well fostered healthy egg development. The embryos showed a healthy eye size and tail shape. On the other hand, the ethanol and nicotine had the expected negative effect on the zebrafish. First, the ethanol seemed to have an equally lethal effect no matter the concentration. This was shown when either two or three embryos survived and hatched in all three concentrations (30 mM, 100 mM, and 300 mM). This was a three fish lower survival rate than

the control group. In Figure 4, a dead egg from 48 hours post fertilization is pictured. The mushy, cloudy texture inside the egg shows the effect the ethanol had on the embryo. To further show the chemical's toxicity, the fish exposed to ethanol had many malformations and birth defects. For example, in Figure 6, a hatched fish with a severely bent spine is shown. However, despite the pernicious effect of the ethanol, there was no major jump in the heart rate data collected from well A4. Yet, this makes sense because alcohol is a depressant that slows down activity. Secondly, nicotine also seemed to have a toxic effect on the zebrafish. Contrary to ethanol, as the nicotine concentration increased, the number of zebrafish deaths increased. For example, while only one fish died in the 0.01 mg/mL well, seven fish died in the 0.2 mg/mL well. In the 0.1 mg/mL concentration, all of the fish died. Furthermore, the zebrafish exposed to nicotine seemed to have many birth defects. For instance, in Figure 3, one hatched embryo had large eyes and showed symptoms of pericardial edema. Also, as shown in Figure 5, some had bent backs. Finally, the nicotine greatly increased the heart rate of the zebrafish. Graph 3 illustrates that while the control fish had a heart rate of 80 bpm, the embryos exposed to the highest concentration of nicotine had a heart rate of 136 bpm. A high heart rate could be unhealthy and dangerous.

Before the experiment, we hypothesized that ethanol and nicotine would have a negative effect on the development of zebrafish. After completing the experiment, we can support our hypothesis for many reasons. First, while only 2 fish died in the control group, the highest number of deaths seen in ethanol-exposed embryos was 8. Furthermore, in one of the nicotine wells, all eight zebrafish perished. Secondly, many birth defects were seen in the ethanol and nicotine embryos. For example, Figure 6 shows a zebrafish with a severely bent back. In addition, a newly hatched fish with pericardial edema was illustrated in Figure 3. Finally, while the ethanol did not increase the heart rate of the zebrafish (it was close to the control rate of 80 bpm), a fish that was exposed to nicotine had a dangerous heart rate of 136 bpm. These results show that ethanol and nicotine were toxic to the zebrafish embryos. From this, it can be concluded that human embryo development would be affected by the chemicals in a similar way. In other words, ethanol and nicotine would also be toxic to developing human fetuses.

Despite the successful results, our experiment had possible sources of error. First, by only using a small sample size, we couldn't control the natural death of some embryos. In other words, some embryos could have died from other environmental factors that were not in response to the chemicals. This could either be fixed by using a larger number of starting fish, or by repeating the experiment many times to see if similar results appeared. Secondly, we could have neglected to remove all of the waste solution after we were finished gathering our daily data. This could have exposed the zebrafish to a greater amount of the chemical leading to an increase in death rate. This could be improved by slowing down when removing the solution and being careful to check for bubbles that were left behind. Thirdly, when removing wastes, we could have either sucked up the zebrafish or pinched them against the well walls. This could have skewed our results by creating malformations in the fish that were mistaken for birth defects caused by the chemicals. To improve this, we would need to go slower and be more careful when changing out solutions and removing waste. It would also help to use a smaller pipette that would not suck up the living fish as easily.

Additional Questions

Questions that emerged from the results of the experiment include testing other species of fish to see if ethanol and nicotine have a similar effect on their embryo development or seeing how small of an amount of various chemicals must a mother ingest to have a negative effect on the development of her child. The latter comes from the modern question of if a small amount of caffeinated coffee consumed during a pregnancy would harm a child.

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