The Effect of Caffeine towards Zebrafish (*Danio rerio*) Juvenile Working Memory Exposed by Unpredictable Chronic Stress (UCS)

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Abstract: The changes in brain structure are caused by dependent and independent factors. Early Life Stress (ELS) is one of the dependent factors that affect the brain's structure and volume. ELS exposure increases synaptic pruning in the neuron in which it disturbs the cognitive function, memory loss, emotion, and risk-taking. Researchers strive to perceive the effect of caffeine in coffee, which can increase memory. Caffeine in coffee has an active compound which increases memory. This study aimed to identify the effect of caffeine in coffee to memory with exposure to stress. The method used in this research was experimental using a post-test with controlled group design. The group were divided into four groups comprising of Negative Control (K1), Positive Control (K2), and group in which exposed by caffeine using different doses (P1 and P2). The exposed group used 20mg/dL and 50mg/dL doses. Hence, the exposure was accorded by the protocol of toxicity for three days. After three days of exposure, group K2, P and P2 were given Unpredictable Chronic Stress (UCS) intervention for seven days. All of the groups were tested using T-maze for three days to see the Zebrafish's memory. Data analysis were collected from Zebrafish time and selection of colour on every side of T-maze. This study shows that there are differences in each group according to time, the average group of K1 (27,11 s), K2 (41,01 s), P1 (25,62 s), P2 (49,22 s). The fastest time was shown in P1. Meanwhile, the slowest time was shown in P2. The test using Shapiro-Wilk showed (P=0,000) p>0,005 in which the data were not well distributed. The data normality using Kruskal-Wallis showed (P=0,000) p<0,005. Hence, H1 can be accepted. Post-hoc test for every group according to time showed group P1-K1, P1-P2, K1-K2, K1-P2 (p<0,005), whereas K1-P1 and K2-P2 (0,063) (p>0,005). Otherwise, compared group according to colour using Chi-Square showed a significant difference (p<0,005). As a conclusion, study report the exposure of caffeine and intervention of UCS in Zebrafish affect memory in the T-maze test.

1 INTRODUCTION

Changes in brain structure are essential in adaptation for the positive or negative external stimulation. A baby's brain structure develops very complexly and matures depending on the environment, education, social interaction, and experience (Elston, 2014). One of the factors in brain development is influenced by a positive environment which leads to resilience. Several kinds of research presented discrepancy in maternal, leading to a cognitive abnormality in children (Weinstock, 2008). It Is necessary to concern parents' education because parents play an essential role in stimulating the children's brain development regarding social and behaviour (Mychasiuk, 2012).

Embryologically, the brain develops in week three. It happens when telencephalon becomes the frontal cortex. The primitive cell is located in the subventricular zone, including axon and the dendritic cell. It proliferates and migrates to the target cell to produce a new layer of the cortex: the afterbirth, post-natal neuron forms sensory motoric and cognitive-based on the stimulation provided. The neuron which is connected well may increase dendritic sprouting and synaptogenesis. Meanwhile, neurons are not connected and used to synaptic pruning and depletion in the cortex (Mychasiuk, 2012).

The stimulation factor in post-natal should be considered as an effect in changing the brain structure by neurogenesis. First, the independent factor is

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caused by genetics in which the neuron is formed based on the image of each other's gene. Second, the dependent factor is created by externals providing stimulation or enrichment of experience after birth (Mundkur, 2005). An environment was rich in cognitive, social and motoric increases brain volume, especially in the prefrontal, hippocampus, and striatum neurons. However, in the form of stress, a hostile environment decreases brain volume with low cognitive function.

Several research pieces have examined using mice by exposing Early Life Stress (ELS) in juveniles as a result of a brain volume decrease. These changes are involved in neuron restriction in ELS exposure which produces neuron restriction through synaptic pruning. The restrictions reduce emotion, cognitive, memory, and decision making (Rocher, 2004; Mundkur, 2005). Another research which concerns on stress pre-natal and post-natal restrict in neuron and reduce cognitive function. PFC has an essential role in stress, especially in the Pyramidal neuron (Kolb, 2017).

The effect of ELS in the restriction on pyramidal neuron PFC correlates in Major Depressive Disorder (MDD), Post-Traumatic Disorder (PTSD), verbal, decision making, loneliness, and anxiety (Rocher, 2004) (Frodl, 2010). The longer the neuron's stress exposure, the easier the neuron restriction in pyramidal apical neuron PFC, presented in several studies (Radley, 2005; Anderson, 2019). It is indicated by evaluating the pyramidal neurons in stress cognitive used in the Y-maze trial. The result showed that there was a difference in the first trial and second. Stress method used other problems, for instance, forced-swim test, elevated plus maze, etc. (Anderson, 2019).

Zebrafish has an analogy brain structure similar to the human brain, especially in memory and cognitive. Human has PFC, which functions as cognitive and long-term memory which is located in the pallium. The Zebrafish analogue brain's research in the pallium is activated when mental plays a role.

Stress affects motoric and behaviour to respond in Zebrafish (Parker, 2013; Anderson, 2019). In neuronal histology, there are changes in mitochondria optic tectum following stress exposure in Zebrafish. In stress, neuron situation restricts where it can be applied with the neuroprotectant use. Coffee is one of the choices for natural neuroprotectant because coffee has caffeine content. The function of caffeine is to reduce the apoptosis in the neuron cells. Research has revealed that coffee contains caffeine used to reduce neuron apoptosis with hampering calcium release and decrease Reactive Oxygen Species (ROS) (Camandola, 2018).

A thing differentiating this research from another research is this research focused on zebrafish memory. This research aims to identify caffeine's effect on Unconditional Chronic Stress (UCS) in zebrafish memory using T-maze. Furthermore, the objective of this research is also to gain a preventive against neuron restriction significantly. How early life stress can affect the development memory.

2 MATERIAL AND METHODS

2.1 Animals and Housing

Juvenile Zebrafish with the age of 30 days (wild type-both sexes) was obtained from Institut Pertanian Bogor (IPB) and was sent to the animal laboratory (pharmacology department–Universitas Islam Indonesia). The aquarium maintained in water quality was based on the instructed protocol. Fish were fed two times a day with micro fish food. The fish were kept in a 1L tank with 40 x 20 x 10 cm^3. The water quality of fish was maintained in 28^oC, pH 7.4 and with water mineral below 800. Biological time for fish was preserved at 7.00 AM – 7.00 PM, and the light was dark. The experimental procedure was approved by ethical clearance in Universitas Gadjah Mada.

2.2 Caffeine Exposure

The caffeine group was exposed by 20mg/dL caffein in group 1(P1), and 50mg/dL caffein in group 2 (P2). A group with exposure 20 mg/dL and 50 mg/dL caffeine extract administered the protocol toxicity in a range of 96 hours. The fish were initially held in a tank with two fish. After 96 hours exposure of caffeine group, P1 dan P2 was given a UCS. We were trying to modify the method from several studies as by (Kim, 2017). The other group was not assigned caffeine, the negative control group (K1) which is only tested with T-maze trial. Then, a positive control group (K2) was only given Unconditional Chronic Stress (UCS) for seven without caffein.

2.3 Stress Exposure

Stress exposure was given to the K2, P1 and P2 group for seven days. UCS was given to the fish which were different in a day. There were restrain stress which the fish are in centrifuge tube 4ml in 90 minutes. Predator exposure, this method was administered by putting the fish beside the predator with a divider. Low water level housing tank, this method decreased water until

dorsal fin of the fish in two minutes. Chasing animals were applied for eight minutes using chaser. Moreover, the tank water replacement method was performed in changing zebrafish tank with new water three times when the zebra must adapt in freshwater. UCS exposure is the same in every group to prevent the different outcome of stress.

2.4 T-Maze

The T-maze test was applied three times in 96 hours where training was modified by the study (Hieu, 2020; Kim, 2017) research by (Hieu, 2020). Fishes were individually transferred to T-maze and observed in one minute. Then, every group was noted for time and chosen arm colour in T-maze as statistical analysis. There were two red and green arms. When fish got into the red component, caught by a net for 30 seconds got punishment. However, in the green arm, the Zebrafish got a reward of fish food. Furthermore, isolated in a green tank, the fish remembered the colour. The training which was provided for the fish aims to increase neuroplasticity memory.

2.5 Statistical Analysis

Data were evaluated using IBM SPSS 24 in searching for homogeneity, normality. The data normality using Shapiro-Wilk analysis (p=0,00) could not be used; the data were not normal. Meanwhile, using Kruskal-Wallis analysis (p=0,00), the hypothesis was accepted. With this average of time, there were significant differences in every group.

3 RESULTS

3.1 The Difference with an Average Time

Table 1: difference with average time according to reaching the target arm using T-maze

no.	Groups	Time average	SD	p
1	[a]K1	27,11	21,88	0, 000
2	[b]K2	41,01	19,61	
3	[c]P1	25,62	18,76	
4	[d]P2	49,22	15,24	

Notes
[a]K1: Negative control group
[b]K2: Positive control group
[c]P1: Caffeine Exposure 20mg/dL
[d]P2 Caffeine Exposure 50mg/dL

Table 1 shows the average time with the slowest time to reach the T-maze arm, which is P2. Meanwhile, the average time of the fastest one is P1. As for time average of training from the beginning until last, the average of time is K1 (27,11±21,88 second, K2 (41,01±19,61s), P1 (25,62±18,76s), and P2 (49,22 ±15,24 s). The fastest to slowest in reaching the T-maze arm are P1, K1, K2, and P2.

Table 2: Kruskal-Wallis group according to time

Relation between groups		p
[a]K1	K2	0,001
	P1	0,855
	[d]P2	0,000
[b]K2	P1	0,001
	P2	0,063
[c]P1	P2	0,000

Notes
[a]K1: Negative control group
[b]K2: Positive control group
[c]P1: Caffeine Exposure 20mg/dL
[d]P2: Caffeine Exposure 50mg/dL

The values of average time are presented in table 2. The post-doc Mann-Whitney test showed significance within the group in K1 with K2 (p=0,00) p<0,005. Group K1 with P1 is not statistically significant (p=0,85). Group K1 with P2 has statistical significance (p=0,00). Meanwhile, K2 with P2 was not statistically significant (p=0,06). Group P1 with K2 is of statistical importance (p=0,00). P1 with P2 shows statistical significance (p=0.00). The significance result shows in the group are P1 with P2 and K1 with P2. Mann-Whitney Test indicated group with exposure of 50mg/dl caffeine which possesses an effect higher time average across the group. However, the group with 20mg/dL is the best time average compared to another group. Therefore, caffeine dose impacts UCS.

3.2 Colour Difference

Table 3. T-maze arm colour is chosen difference in every group using Pearson Chi-Square

	Arm Chosen	K1[a]	K2[b]	P1[c]	P2[d]	Total	p
Colour	Not choosing	9	18	5	25	57	0,000
	Green	28	16	30	12	86	
	Red	8	11	10	8	37	
Total		45	45	45	45	180	

[a]K1: Negative control group
[b]K2: Positive control group
[c]P1: Caffeine Exposure 20mg/dL
[d]P2: Caffeine Exposure 50mg/dL

The analysis of colour difference across the group using the chi-square method with two variables was nominal categorical. The values of colour difference presented in table 3 show K1 group 9 Zebrafish preferred not to choose red arm colour; 28 Zebrafish decided green arm colour and 8 Zebrafish chose a red arm colour. K2 group 18 Zebrafish picked not to choose arm; 16 Zebrafish chose green arm colour, and 11 Zebrafish preferred to select a red arm colour. P1 group 5 Zebrafish liked not to choose arm; 30 Zebrafish chose green arm colour, and 10 Zebrafish chose a red arm colour. However, the P2 group 25 Zebrafish decided not to select arm; 12 Zebrafish chose green arm colour, and 8 Zebrafish chose a red arm colour. The comprehensive training for T-maze is three.

Table 3 presents a group for P2 as the highest for not choosing any arm. The tallest red arm colour is the K2 group, although the highest green arm colour is the P1 group compared to the other groups. The analysis result shows a significant difference between the groups in choosing the arm colour (p=0,00). Clinical analysis indicated for memory in Zebrafish is based on the colour selected for each group. Zebrafish which chose green colour received a reward. Otherwise, Zebrafish which chose red colour received a punishment of stress exposure.

4 DISCUSSION

The effect of caffeine on zebrafish behaviour has been extensively studied using toxicity. Stress and caffeine which have been applied to the fish, have a variation on motoric and memory. There is a difference in the behaviour given with high dose (50mg/dL) and low dose caffeine (20mg/dL). This study shows the use of low dose caffeine which can control stress and increase the memory. Based on Zebrafish's research (Adlioglu, 2012; Ruiz-Oliveira, 2019), the low dose of caffeine (5-140 mg/dL) may increase memory motility cognitive.

Furthermore, a study in mice using 20mg/dL caffeine dose reveals an optimal result in the forced swim test. Therefore, in this study, we used 20mg/dl dose to Zebrafish to reach the optimal memory. The increased memory performance by caffeine is related to its effect in increasing Acetylcholine (Ach) which can inhibit the oxidation in a neuron. Ach affects in expanding the work of GABA-Benzodiazepine, where GABA works to inhibit glutamate. Furthermore, caffeine occurs 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydro-Pyridine (MPTP) functioning as a glutamate inhibitor.

Otherwise, the fish which was not given with caffein reduced movement, memory and aggressive cognitive. It was also followed by UCS cognitive change in Zebrafish. Chronic stress affects restriction in the pyramidal neuron. Restriction in pyramidal neuron reduces regulation on top-down control in working memory dominated in emotion. When Zebrafish were exposed by UCS (K2) and had been tested, it did not improve for choosing T-maze arm (Arnstern, 2015; Goldwater, 2009; Mizoguchi, 2003). Anatomically, PFC in Zebrafish is related to pallium. It controls the cognitive in the Zebrafish, and sub-pallium works as memory similar to the amygdala and hippocampus in humans.

Stress activates HPA-Axis stimulated by catecholamine and corticosteroid in the amygdala. In this situation, stress inhibits frontal cortex in which PFC functions as cognitive and working memory. Based on this study, the group exposed with UCS (K2) reduced in movement and was inhibited in choosing arm colour, in which time to reach the T-maze arm was also slower presented in table 1 and table 3. However, a group which was not exposed to stress exposure (K1) was faster in choosing a T-maze arm. This study has shown that memory and decision depend on stress stimulation from catecholamine and glutamate. Meanwhile, it affects the working memory in Zebrafish (Haight, 2011).

Caffeine is a substance that reduces neuron restriction in the enhancement of memory and decision. Low dose caffeine is a more effective substance which is shown in table 3 with the T-maze test. This study indicates a similar result with study in (Adiloglu, 2012; Ruiz-Oliveira, 2019). In Ruiz-Oliveira et al. (2019), Zebrafish with low dose reached arm target faster than the higher amount. The P1 group has a similar average time in choosing a T-maze arm compare to K1. Low caffeine substance proved that stress exposure did not affect Zebrafish, which were given caffeine in low dose. The consumption of low caffeine dose decreases in anxiety by inhibiting the work of *A2a adenosine receptors*. Otherwise, the P2 group had a disruption in memory. This response induced zebrafish freezing and continued exploring the tank without choosing a T-maze arm (table 3). It can be interpreted that a high dose of caffeine may escalate anxiety and stress. Based on the study (Ruiz-Oliveira, 2019; De Carvalho, 2019) in Zebrafish, the psychoactive and dependent fish were given 50mg/dL caffeine dose. Based on the FDA, the consumption on caffeine 400mg/dL disrupts adults' health.

The training was conducted to increase the memory in neuroplasticity and a new memory.

However, the more Zebrafish are trained, the more neuroplasticity makes recent memory. In this case, pyramidal neurons function as working memory and higher cognitive function (Arnstern, 2019).

Cognitive and memory may decrease when the neuron's restriction of repeated stress and a higher dose of caffeine cause anxiety. The limitation in the neuron is caused by glutamate exposure in the apical neuron. It affects restriction dominated in the apical neurons. The layer in the apical neurons becomes dominant due to Excitatory Post Synaptic Currents (EPSP). Neuron Restriction is caused by how long neurotransmitter exposure in glutamate stands towards the other neuron. It is called Long Term Potentiation (LTP). LTP affects Spike timing-dependent (SDPT) by glutamate exposure and increases in calcium for cell apoptosis. The longer the LTP is, the more neuron restriction happens. Therefore, the relation between time and choice of T-maze arm is related to the exposure of UCS and caffein exposure presented in table 2 and table 1. Stress situation, the neurotransmitter focuses on the bottom-down in the amygdala in which pyramidal neuron restricts. Meanwhile, K1 and P2 group have better working memory.

5 CONCLUSION

Caffeine affects memory and stress. Caffeine may reduce stress at a low dose. Moreover, caffeine affects the memory in Zebrafish.

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