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ORIGINAL PAPERS

Behavioural and Metabolical Changes Associated with the Pathophysiology of Alzheimer's Disease in Zebrafish

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Abstract

Background. Affecting 60% of the people diagnosed with dementia, Alzheimer's disease is a neurodegenerative pathology that negatively impacts the cognitive function. It is characterised by symptoms as memory loss, locomotor difficulties, behavioural changes, and even rationalization issues. This disease has been studied on both rodents and fishes. Rodents helped science people establish the basic neurobiology of dementia, while fishes (*Danio rerio* – the zebra fish, especially) were more appropriate as transgenic models. Recent studies proved that transgenically induced Alzheimer's disease at zebra fishes is not defined only by cognitive decline but also by motor function disorders. **Objectives.** This study aims to analyse how iron chloride and sucrose impact zebra fishes' locomotion and memory by using the T maze. **Methods.** 50 zebra fishes were purchased from a local pisciculturist and randomly divided in 4 experimental groups. They were accommodated in the Ecotoxicology laboratory at "Alexandru Ioan Cuza" University for 3 weeks according to the European Union Commission and European Union Council recommendations regarding experimental purposes animals' protection and accommodation. Fishes' possible behavioural changes were analysed after they were given sucrose and iron chloride separately and in their combination. **Results.** We observed that sucrose administration negatively impacted the locomotory activity while iron chloride surprisingly increased it. The possible explanation is the fact that iron chloride generated anxiety - an early-stage Alzheimer's disease

symptom, and therefore improved fishes' swimming performance. Successive sucrose and iron chloride administration also led to increased locomotor activity.

Keywords: *Alzheimer's disease, dementia, zebra fish, behaviour, sucrose, iron chloride*

Introduction

In 1910, the term 'Alzheimer's disease' was used for the first time, naming the neurodegenerative disease after Alois Alzheimer – German psychiatrist and neurologist. Initially, this pathology was described as an 'unusual disease affecting the cerebral cortex', observed at a 51-year-old female patient who had symptoms such as: memory issues, behavioural changes, difficulties in doing usual activities and other symptoms that were not noticed before at young people, comparing to elders who had dementia. Years later, after the patient's death, the autopsy proved that there was massive brain damage, especially in the parietal and frontal regions (Hippius & Neundorfer, 2003).

Known as the 21st century disease, Alzheimer's disease is represented by a neurodegenerative process that slowly contributes to the loss of the cognitive function, leading to the patients' death. It affects around 60% of the people suffering with dementia, being specific mostly to elders, although young people tend to be diagnosed with it as well (Marica, 2011).

The main risk factor generating Alzheimer's disease is the presence of apolipoprotein E (APOE) allele. Out of its 3 forms: E2, E3 and E4, E4 type is considered to be involved in Alzheimer's disease pathophysiology, judging by the fact that it APOE has important functions such as: motor coordination, cholesterol distribution, growth, repair and maintenance of myelin sheath and brain cells. There are 9% chances that an individual that lacks E4 allele has Alzheimer's disease, while the risk rises to 29% for people who have it (Cummings & Cole, 2002). Besides repeated, serious cranio-cerebral trauma, other risk factors in developing this disease could be biological: low levels of folate and vitamin B12, high levels of homocysteine and cholesterol, type 2 diabetes, hypertension, and social factors: poor education and low income for example (Marica, 2011) (Apostolova, 2016).

Alzheimer's disease early clinical examination is characterized by symptoms as apathy and generally decreased interest or concern. Around 50% of the patients also have depression related symptoms and 25% can hallucinate, while mobility abnormalities are observed during the last phase of the disease (Cummings & Cole, 2002). According to a 2019 study, patients' symptoms vary with the disease phase as it follows: early stage – organizing issues, forgetting dates, names, details about him/herself, getting lost in familiar places; middle stage: hallucinations, compulsive behaviour, anxiety, reading and writing issues,

sleeping disorders; late stage: personality and behavioural changes, trouble maintaining a conversation, difficulties in moving, eating, and swallowing, lost control of bladder and gut. Studies suggest that Alzheimer's disease has a long-term preclinical stage where body's functions decrease. However, its symptoms are insufficient to diagnose dementia. An increasing number of studies claim that there is a connection between noncognitive symptoms such as loss of locomotion and the early stage of Alzheimer's disease (Buchman & Bennet, 2011). Muscular volume is correlated with this pathology both for individuals who have dementia and for the ones who do not. High levels of physical fragility before death are linked to high chances of discovering Alzheimer's disease signs at autopsy (Buchman et al, 2008).

Macroscopical examination proves that Alzheimer's disease leads to brain morphological and chemical changes. It generates cerebral cortex atrophy associated with decreased volume of parietal, temporal, and frontal gyri. Microscopical analysis of the brain at autopsy shows visible senile plaques between neurons, formed by beta-amyloid build-ups - protein fragments accumulated at the presynaptic terminal, generating insoluble masses that prevent neural communication. Therefore, it is believed that Alzheimer's disease is the result of the disturbed balance between production and excretion of amyloids (Marica, 2011). Thus, Alzheimer's disease diagnosis methods include examination of clinicals symptoms, biomarker tests, and, nonetheless, amyloid positron emission tomography – able to detect the neuropathology at microscopical levels (Apostolova, 2016).

Another characteristic of the Alzheimer's disease is iron homeostasis disturbance. Excessive iron stimulates beta-amyloid build-up and, therefore, neurofibrillary tangles. Iron levels increase gradually, with age and, by magnetic resonance imaging, it was possible to discover that iron levels are surprisingly high in the cortex of patients suffering from Alzheimer's disease and, therefore, iron became extremely important when making the diagnosis (Du et al, 2018).

This study aims to analyse how iron chloride induced Alzheimer's disease could possibly affect zebra fishes' behaviour.

Zebra fish as animal model in Alzheimer's disease research

Zebra fish – *Danio rerio* is one of the most important animal models used in genetical, neurophysiological and biomedical studies due to it has a series of advantages for research: small size, repeated reproduction during a single year and new generations once every 3-4 month, facts that make them ideal for selection. Biomedically, this species is used mainly to study human diseases and for drug tests.

Studies regarding these fishes' behaviour have been done over the past years, describing their aggressivity, dominance, exploring and, especially,

cognitive behaviour. Both zebra fish sexes can establish dominance hierarchies and aggressive interactions between individuals could involve even bites – important aspects in reproductive and eating behaviour (Grant & Kramer, 1992). The exploring behaviour is also essential as it increases the probability that individuals detect and avoid predators (Pitcher & Parrish, 1993). Regarding cognition, learning is critical in zebra fishes' behaviour, as it is important for the social interaction, eating and predator avoidance. The learning process can be studied by using a condition model, where the fishes need to swim in a specific direction in order to obtain food (Bilotta et al, 2005).

Zebra fish has also been used to study different degenerations occurring during the aging process: cardiovascular changes, low levels of neurogenesis, learning issues and decrease of cognitive function (Keller & Keller, 2018). According to some studies, along with the aging process, zebra fishes' resistance and speed diminish as the young individuals' swimming performance improves (Gilbert et al, 2014).

Danio rerio can be used in studying different types of central nervous system pathologies like cancer, epilepsy, and anxiety disorders. It is also useful in pharmacological screening development (Gerlai, 2003) and embryo development studies (Blaser & Gerlai, 2006). Various human neuropsychiatric disorders have been studied both using rodents and zebra fish models: schizophrenia, bipolarity, anxiety, and depression are just few of them (Kalueff et al, 2014).

Even though the rodent animal model had a major importance for science by giving information about the way drugs could work in Alzheimer's disease treatment and about the neurobiology of dementia, this is not actually frequently used nowadays (Hargis & Blalock, 2017). Zebra fish has the great advantages that it has easily quantifiable behaviours, a simple nervous system and completely sequenced genome - fact that allows comparisons between its nucleotide sequences and another animals'. Furthermore, its hematoencephalic barrier is functional 3 days after fertilization, which makes it possible to realize a pharmaceutical screening similar to the one related to more complex vertebrates (Jeong et al, 2008). Also, zebra fish possesses 84% of the human dementia related genes (Howe et al, 2013).

Alzheimer's disease symptomatology in humans and zebra fishes

People diagnosed with Alzheimer's disease seem to have difficulties regarding memory, attention, motor coordination, equilibrium and even feeding. They could also develop anxiety, sudden mood swings, sleeping disorders or hallucinations (Voisin & Vellas, 2009). The zebra fish model involves many of the symptoms specific to humans. In order to understand the way this is possible, neurologic structures of this animal model have been studied over the past years, concluding that, even though their nervous system is not identical to humans',

zebra fishes' encephalic structure is similar to ours (Lieschke & Currie, 2007). Due to this similarity, Alzheimer's disease symptoms could be studied using the transgenically induced disease method (Nery et al, 2013). Sick fishes' behaviour was studied by analysing their reactions to different stimuli. For example, healthy individuals would react by shaking as a response to an acoustic stimulus. On the other hand, sick fishes easily accommodate with it and physical reactions decrease (Thomson & Spencer, 1966). In this context, drugs considered potentially impactful in treating Alzheimer's disease (like acetylcholinesterase inhibitors) were administrated to establish their efficiency. As a result, the accommodation to sound exposure diminished and mobility as a response to acoustic stimuli increased (Best et al, 2008).

Materials and methods

In order to analyse the research hypothesis, 50 zebra fish individuals were purchased from a local pisciculturist and accommodated in the Ecotoxicology laboratory at "Alexandru Ioan Cuza" University for 3 weeks. There was respected every 18th July 2007 recommendation from the European Union Commission regarding the accommodation of organisms for experimental purposes and every 22nd September 2010 recommendation from the European Union Council regarding science purpose animals' protection. Furthermore, the study was approved by the Faculty of Biology's Ethics Commission. The animal study group was kept in an aquarium characterised by the following parameters: temperature - approximately 27 degrees Celsius, pH – 7,5, oxygen level – 7,20 mg/L, lightning – white light led band that maintained the circadian rhythm at a 14:10 ratio (light:dark). Daily, the water was changed, and the fishes were fed with TetraMin special flakes. Conditions were established based on Reed et al, 2011 article.

Iron chloride was purchased from Emsure, 10025-77-1 250g, Merek, Darmstadt, Germany, and commercial sugar was used as sucrose source. These chemicals were administrated by dissolving them in the aquarium on a daily basis in order to make sure that they are present in the fishes' habitat in the needed concentration. Tests included the usage of 0,1 mg/L FeCl3, 83,25 μM sucrose, and their combinations.

Regarding the experimental protocol, adult zebra fishes were randomly distributed in 4 test groups as it follows: group 1= control, group 2= sucrose administration, group 3= iron chloride administration, and group 4= sucrose and iron chloride, successively. For group 4, fishes were fed with sucrose for 4 days to rise their blood glucose level. Every test group accommodated with the experimental conditions for 2 days. Afterwards, every single individual was initially analysed by the performance test in order to observe the pre-treatment behaviour and, the day after, the chemicals were introduced in their habitat. Behavioural data was collected every 24 hours after chemical administration. The

entire experiment ended with the fishes' sacrifice by immersion in iced water according to the 2007 European Union Commission recommendations.

The performance test (the behaviour test) was meant to analyse the effect the chemicals used could have on locomotion. In this context, parameters like the total distance swum and the time spent in the 3 T maze arms were quantified. Every single individual's activity was monitored using a video camera fixed on top of the maze and connected to the computer. Every parameter's value was measured by EthoVision XT 11,5 software in 4-minute-long sessions.

Statistical analysis of the collected data was done by applying the ANOVA unidirectional test, comparing the initial behaviour with the post-treatment one.

Results

Data regarding the distance travelled by every individual over the 4 experimental days shows that there is a major difference between the 4 study groups, and, also between each experimental day. Data obtained is described in the tables and charts below:

Table 1. Total distance swum by the test groups in each experimental dazy

	Pre-treatment	Day 1	Day 2	Day 3	Day 4
Group 1	485.9 ± 60.3 cm	432.2 ± 86.2 cm	279.2 ± 18.08 cm	362.3 ± 35.3 cm	477.9 ± 44.3 cm
Group 2	739.9 ± 77.7 cm	346.7 ± 24.8 cm	364.4 ± 40.5 cm	448.5 ± 50.7 cm	337.4 ± 26.7 cm
Group 3	637.5 ± 46.1 cm	450.0 ± 24.7 cm	432.8 ± 40.6 cm	579.3 ± 31.03 cm	463.1 ± 23.3 cm
Group 4	967.5 ± 62.4 cm	623.9 ± 94.5 cm	701.9 ± 112.1 cm	559.7 ± 48.7 cm	472.8 ± 40.5 cm

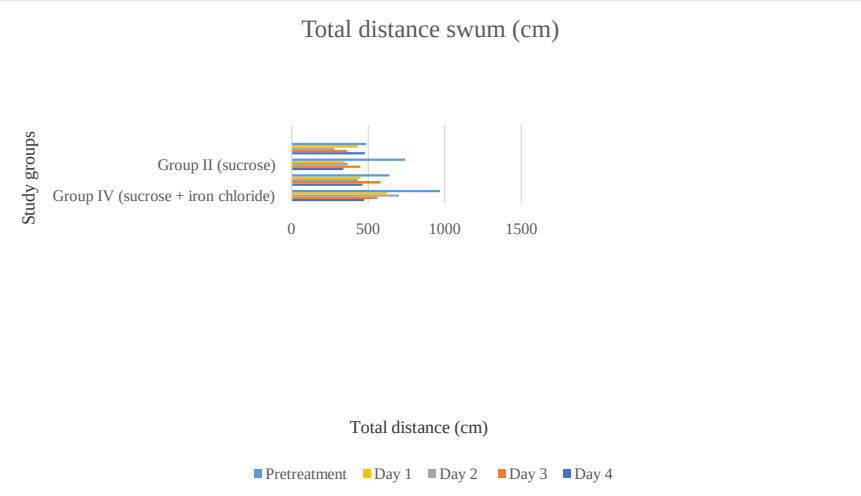

Figure 1. Total distance swum by zebra fishes – comparison between the study groups

There is a major difference between the data obtain for each study group. Fishes that were fed with sucrose swam the shortest distance, while those that were given iron chloride had the best swimming performance – swam the longest distance throughout all of the experimental days.

Table 2. Time spent in the central maze arm by each test group

	Pre-treatment	Day 1	Day 2	Day 3	Day 4
Group 1	147.2 ± 21.7 s	139.7 ± 37.2 s	183.6 ± 21.1 s	168.9 ± 16.8 s	113.2 ± 17.1 s
Group 2	30.9 ± 6.1 s	141.4 ± 26.8 s	64.4 ± 13.5 s	139.1 ± 22.3 s	85.6 ± 30.6 s
Group 3	127.1 ± 17.8 s	184.6 ± 27.3 s	165.1 ± 34.4 s	86.6 ± 36.9 s	132.2 ± 23.3 s
Group 4	32.7 ± 4.9 s	144.9 ± 29.3 s	186.1 ± 18.1 s	159.1 ± 19.9 s	149.5 ± 21.5 s

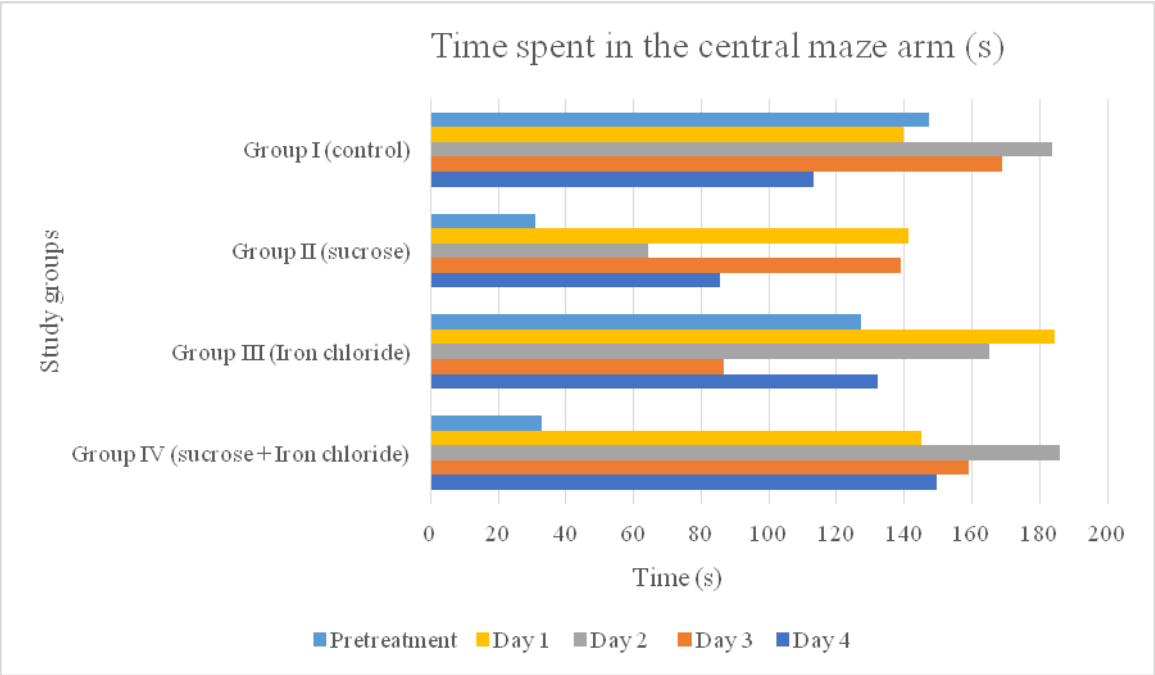

Figure 2. Time spent in the central maze arm – comparison between the study groups

Data is inconsistent, as there are significant differences between the data specific for each of the 4 groups and between the data collected in each experimental day.

Table 3. Time spent the right maze arm by each test group

	Pre-treatment	Day 1	Day 2	Day 3	Day 4
Group 1	48.8 ± 13.7 s	10.4 ± 5.4 s	8.7 ± 4.0 s	34.0 ± 12.3 s	41.7 ± 12.4 s
Group 2	34.9 ± 5.8 s	55.4 ± 16.9 s	105.2 ± 16.5 s	42.3 ± 9.5 s	105.8 ± 30.5 s
Group 3	25.1 ± 5.5 s	19.3 ± 11.1 s	26.9 ± 14.4 s	72.2 ± 10.7 s	51.5 ± 12.2 s
Group 4	24.9 ± 4.1 s	39.8 ± 24.2 s	14.4 ± 8.3 s	26.3 ± 6.5 s	53.0 ± 17.2 s

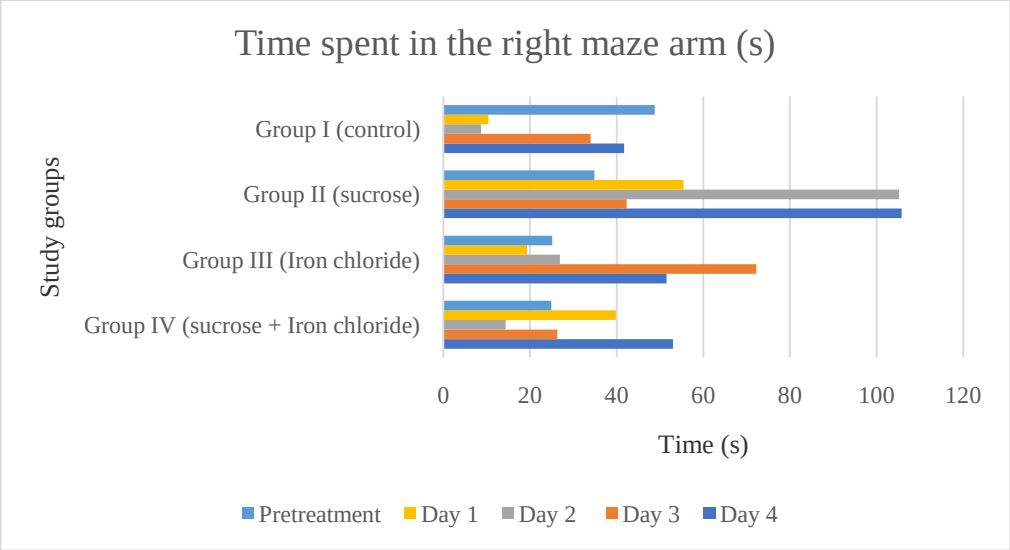

Figure 3. Time spent in the tight maze arm – comparison between study groups

Analysing the above chart, there is a significant difference between data specific to each study group and, also, data varies from one day to another.

Table 4. Time spent in the left maze arm by each test group

	Pre-treatment	Day 1	Day 2	Day 3	Day 4
Group 1	14.3 ± 4.3 s	66.2 ± 32.3 s	31.0 ± 13.8 s	22.4 ± 6.4 s	68.1 ± 18.4 s
Group 2	30.7 ± 6.2 s	31.0 ± 14.4 s	44.6 ± 11.8 s	38.8 ± 10.5 s	32.6 ± 5.74 s
Group 3	61.8 ± 11.7 s	25.0 ± 12.7 s	39.7 ± 20.3s	60.2 ± 24.4 s	39.3 ± 12.3 s
Group 4	47.7 ± 5.0 s	8.6 ± 2.8 s	20.9 ± 7.4 s	35.2 ± 14.5 s	17.5 ± 8.5 s

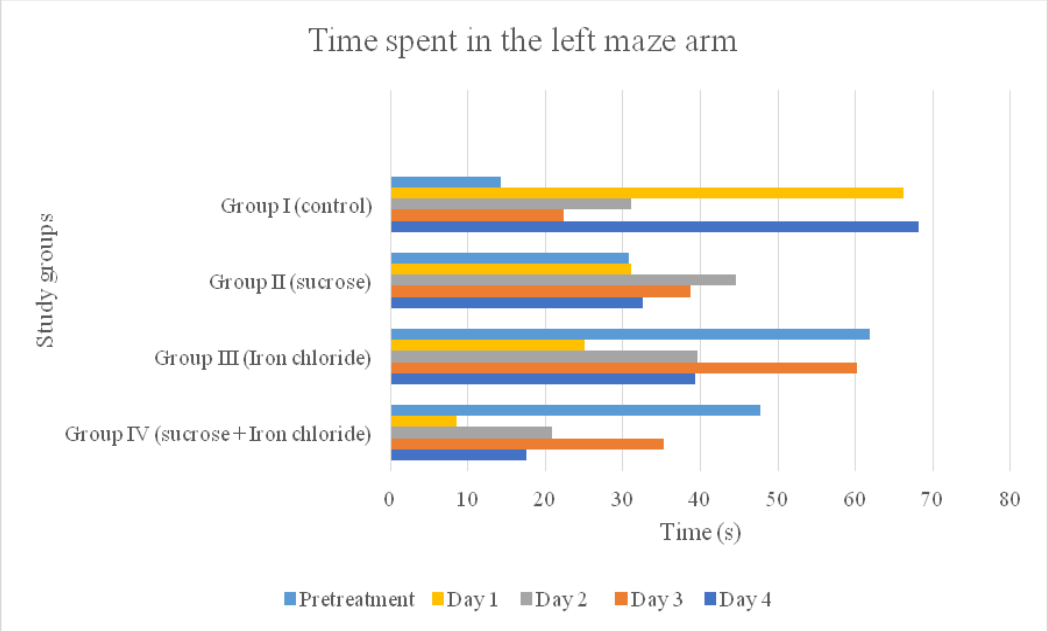

Figure 4. Time spent in the left maze arm – comparison between the study groups

It is visible that results regarding the time fishes spent in the left T maze arm are inconsistent as data is different according both to each study group and to each experimental day.

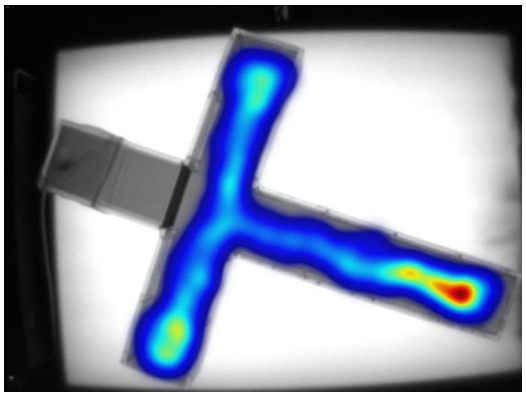

Figure 5. Group 3 (iron chloride), day 4

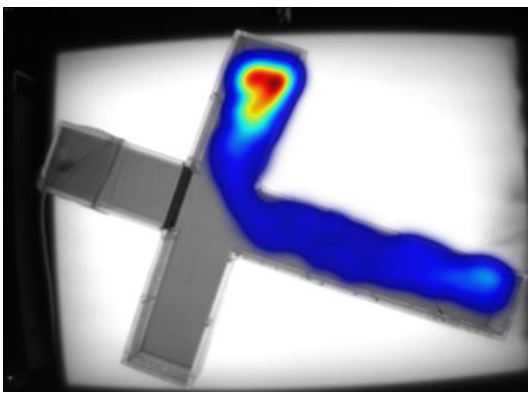

Figure 6. Group 4 (Sucrose+iron chloride), day 4

Thermographic maps show that cognitive function decreases as the fishes tend to neglect some of the maze arms.

Discussions

The way sucrose can affect the total distance swam by zebra fishes was analysed according to the protocol elaborated by Shovit Ranjan & Praveen Kumar Sharma in their 2020 article. Sucrose administration generated a negative impact on the study groups' mobility, decreasing the total distance swum throughout the experimental days. This fact indicated that the sucrose concentration used (83,26 μM) created a metabolic deficiency.

Analysing data obtained from study groups III and IV – the ones that were given sucrose and iron chloride successively, the experimental model was not confirmed. The initial expectations included the fact the fishes would swim a shorter distance, as iron chloride administration has usually neurotoxic effects, leading to cognitive dysfunctions. Surprisingly, the results showed that iron chloride did not have a negative impact on locomotion. According to specialists (Egan, 2009), the possible explanation for long distance swum after iron chloride administration is that it induced anxiety. This hypothesis is based on the fact that there are studies that proved how anxiety is one of the early-stage Alzheimer's disease symptoms.

Furthermore, data obtained shows that the iron chloride only group develops an anxious behaviour, swimming a long total distance in the entire maze, while fishes that got sucrose firstly and iron chloride secondly swam a long total distance but avoided one of the maze's arms. This aspect is considered to be sign of neurodegeneration. Regarding the time spent in all 3 arms of the maze, it was noticeable that there is a tendency of preferring the centre, followed by the right arm, and fishes spent the least amount time in the left arm.

Our experiment also proved cognitive function decrease by comparing the thermographic maps obtained using the EthoVisionXt 1.5 software. These showed that cognition was negatively affected as fishes tended to avoid some of the T maze arms.

Conclusions

Alzheimer's disease is a neurodegenerative disorder with noticeable effects on locomotion. Because of the individuals' memory issues, spatial orientation is difficult, leading to nonsense movements. Taking this into consideration, our study analysed zebra fishes' locomotion changes after sucrose and iron chloride administration. We established the following conclusions:

1. Sucrose administration had obvious effects on the study groups, generating a negative impact on their locomotor activity;
2. Iron chloride administration did not generate the expected locomotor activity decrease, it actually increased it;
3. There were significant effects on locomotion and memory after the successive sucrose and iron chloride administration.

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