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Studying the impact of anthocyanin extract from black rice on regenerative abilities in the zebrafish D*anio rerio*

Abstract. Zebrafish (*Danio rerio*) is becoming more widespread in all types of biomedical researches. Owing to its high regenerative potency, zebrafish can be used in human therapy as a model organism. Anthocyanins, extracted from plants, are presented as a prospective food supplementary, demonstrating anti-bacterial, anti-fungal, anti-inflammatory, antioxidant and tumor-suppressing effects. In this study, we tested the impact of anthocyanins, extracted from black rice (*Oryza sativa*), utilized as a dietary supplement to commercial and live feed, on regeneration of amputated caudal fin in the zebrafish population. 4 months old (120 days postfertilization) newly amputated zebrafish were given anthocyanins daily during 21 days. The main ichthyological parameters, such as total length (TL) and length of caudal fin (LCF) were measured every 7 days. The obtained data was processed via T-test. Despite all predictions, the control group demonstrated better growth and regeneration than the experimental fishes. It might correlate to high concentration of anthocyanins, which in turn activates the process of apoptosis.
Key words: zebrafish, *Danio rerio*, anthocyanins, regeneration, diet.

Introduction

The zebrafish (*Danio rerio*) is a small tropical fish, which became more frequently used as as a model in biomedical researches. Owing to its high potency to regenerate amputated tissues and organs, such as heart muscle, eyes and fins, it can be useful to study the process of renovation in humans. For zebrafish, food and food supplements is an important factor, regulating many inner processes, including its growth and regeneration [1].

Anthocyanins are considered the most important group of pigmented flavonoids with more than 600 compounds identified in nature [2]. They are water-soluble compounds that provide color from red to dark-blue and black to such plant tissues as leaves, stems, roots, flowers and fruits, in dependence on pH of the medium and their structural composition [3]. In fact, there are about 25 various aglycones, detected in nature now. However, only six anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin) are widespread in plants, accounting for more than 90% identified anthocyanins [4-6].

The relative abundance of anthocyanins may vary according to the species depending on external and internal factors. Genetic factors and agronomic practices, intensity, processing and storage conditions influence the level of anthocyanins. Among the most common anthocyanidins in the higher plants, the glycosides of the three non-methylated anthocyanidins (cyanidin, delphinidin and pelargonidin) are the most abundant in nature, 80% of leaf pigments, 69% of fruits and 50% of flowers. The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%) and malvidin (7%). The most widespread anthocyanin in fruits is cyanidin-3-glucoside. Recently, anthocyanin composition and its antioxidant capacity were determined for highly pigmented edible vegetables [7-9]. Consistently, fruits contain anthocyanins which indicate the best natural source of these compounds. Red and blue highly pigmented fruits, mainly berries such as blueberry, blackberry, blackcurrant, cherry, cranberry, raspberry and strawberry fruits, have been comprehensively analyzed suggesting that anthocyanins contribute significantly to the antioxidant activity.

Interest in studying a diet rich in polyphenols, which includes anthocyanins was intensified after identifying their potential health benefits [10]. It has been detected, that high consumption of anthocyanin-rich foods might provide potential benefits on the health of people, suffering from cancer, aging, obesity, neurological diseases, inflammation, diabetes, and bacterial infections [11-13].

Anthocyanins as food supplements also have a huge impact on health and help to fight with obesity, diabetes, cardiovascular, respiratory and extractor diseases, improve mood, digestion, regeneration and so on. A large list of experiments revealed the anti-diabetic properties of anthocyanins, and simultaneous effects of these biologically active compounds, including decrease in blood glucose, preventing the production of free radicals, increased pancreatic insulin production and improvement of insulin resistance.

Materials and methods

Danio rerio husbandry. The main principles and protocols of breeding and housing of zebrafish were borrowed from "The Zebrafish Book", monograph by Westerfield. The whole population of adult *Danio rerio* was kept at 23 °C, with pH 7 ± 0, and saturation level >90%. The photoperiod was 12L: 12D. An artificial source of light was used during the experiment. The volume of both tanks was equal to 200 liters, what helped us to minimize the negative effect of overpopulation (maximum 135 fishes/tank). To maintain water quality, 10% of water was exchanged every week. For the experiment zebrafish both males and females about 120 days-old were collected from the Laboratory of Aquaristic at al-Farabi Kazakh National University (KazNU) for amputation (dfa). For amputation only healthy fishes without any visible marks of diseases were selected.

Procedure of caudal fin elimination. Before amputation all fishes were anesthetized in 2 mL 1% lidocaine per 100 mL of water during 2 min. Caudal fin was amputated totally by a sharp razor. After the procedure zebrafish were divided into control and experimental groups and returned to the tanks, containing fresh, warm and oxygenated water. All stages of the experiment were performed fully according to the rules and standards accepted in modern practice.

Anthocyanins extraction. Black rice *(Oryza sativa)* was selected as a source of anthocyanins. To check various solvents and their ability to extract anthocyanins, distilled water, isopropyl, ethyl and methyl in the presence of HCl in different concentrations were used as a source. 2.5 g of raw material were put in a blender and crushed into homogeneous powder. Obtained substance was dunked in 25 mL of extractant and incubated for 1, 2, 3, 4, 8, 24 h under the general room temperature as well as in water baths: LOIP (Japan), IKA-WERKE basic pro 20 (Germany).

Then, 1 mL of solution was centrifuged at 10 000 rpm within 3 min, supernatant was removed, and the pellet was diluted 100 times. The optical density was measured by V-3000PC Spectrophotometer (Austria) at 538 nm. The blank corresponded to the extractant. For the further utilization of extracts of anthocyanins *in vivo* it was essential to remove the extractant. The solution was evaporated in a vacuum rotary evaporator BUCHI Rotavapor R-124 (Switzerland) at 55°C during several hours to delete any liquids. Then the pellet was suspended with100 mL of distilled water, mixed and kept in the refrigerator.

Anthocyanins assessment. In the international scientific practice, it has been generally accepted the method of measurement of anthocyanins concentration at different pH. Moreover, the exposure time must be in the range of 15-60 min to exclude overestimated results. Buffer solutions were prepared according to the following protocol:

Solution A: 0.025 M KCl, pH 1.0

A powder of KCl weighing 0.465 g was dissolved in 240 mL of distilled water in a beaker. The pH was adjusted to 1.0 with a solution of concentrated hydrochloric acid, adding drop by drop. The resulting solution was transferred to a volumetric flask with a capacity of 250 mL and brought to the mark with distilled water, followed by pH control.

Solution B: 0.4 M CH_3COONa, pH 4.5

A weighed portion of CH3COONa · $3H_2O$ weighing 13.6 g was dissolved in 240 mL of distilled water in a beaker. The pH was adjusted to 4.5 with a solution of concentrated hydrochloric acid, adding it dropwise. The resulting solution was transferred to a 250 mL volumetric flask and adjusted to the mark with distilled water, re-monitoring the pH.

Aliquots of the analyzed anthocyanins Va extract (with preliminary dilution selection) were transferred to Vk volumetric flasks and adjusted to the mark with solutions A and B, respectively.

The calculation of the concentration of anthocyanins was performed according to the formula:

$$(A \times MW \times DF \times 1000) / (\varepsilon \times 1),$$

where $A = (A_{520} - A_{700})$ pH 1.0 – $(A_{520} - A_{700})$ pH 4.5;

MW – molecular weight 449.2 g/mol (cyanidin-3-glucoside);

DF – number of dilutions;

ε – molar absorption coefficient 26,900 L/mol.

Behavioral extinct assessment. To assess fish behavior before and after caudal fin amputation, physical and psychological conditions, classical methods such as swimming test, light reflex, Pavlovian conditioning and anxiety test were used.

Forage preparation. To check the activity of extracts of plant anthocyanins, 150 g of frozen Aqua menu artemia (*Artemia salina*) and 100 g of tubifex (*Tubifex tubifex*) (JBL NovoFex, UK) were soaked in 100 mL of water extract of black rice for 24 h and given with Betta Menu (Tetra, Germany) to the experimental group once per day during 21 days. The control group was fed by a mix of natural and commercial food in equal proportions.

Statistical analysis and modelling. All analyzes were performed via Statistica Software (StatSoft, Russia), all graphs were generated by Excel Software. Every week the major ichthyological parameters as TL – full body length and LCF – caudal fin length were performed. To process obtained data the method of T-test was selected. To calculate it, the following formula was used:

$$t = \frac{M_1 - M_2}{\sqrt{m_1^2 + m_2^2}},$$

where M_1 and M_2 – the arithmetic mean of the first and the second groups;

m_1 and m_2 – the mean error of the first and the second groups.

All calculations were performed by Statistica software.

Live imaging. To take pictures in high resolution, the fishes were fixated in glycerol. All images and videos were obtained by Motic DM143 series and Motic Images Plus 3.0 software (Motic, China).

Results and discussion

As in alcohol homologous series the effect of extraction grows from isopropanol to ethanol and methanol, it has been decided to extract anthocyanins from black rice (*Oryza sativa*) in various spirits (Figure 1). Distilled water, isopropyl, ethyl and methyl in different concentrations were used as solvents.

a b

Figure 1 – *Oryza sativa* as a source of anthocyanin extracts.
Note: A – raw material (x10) and B–rice powder (x10)

Isopropyl appeared not appropriate to use for anthocyanin extraction because of its low reactivity (Table 1). Maximal optical density (OD) 0.0034 at λ_{max}=538 nm was approached only after 24 h from the start of extraction, whereas in the tube with ethanol higher OD was observed in 1 h, and in methanol this indicator was 2.5 times higher. Even extraction in distilled water demonstrated more significant ability to extract anthocyanins.

Table 1 – Usage of different alcochol doses in order to extract anthocyanins, OD at λ_{max}=538 nm

Extractant	1 h	2 h	3 h	4 h	6 h	8 h	24 h
Distilled water	0.012	0.029	0.017	0.055	0.013	0.009	0.003
1% isopropanol	0.009	0.011	0.012	0.014	0.024	0.026	0.034
2% isopropanol	0.002	0.006	0.015	0.018	0.011	0.010	0.007
5% isopropanol	0.004	0.005	0.011	0.013	0.010	0.007	0.003
1% ethanol	0.042	0.061	0.067	0.092	0.103	0.106	0.099
2% ethanol	0.058	0.078	0.092	0.076	0.065	0.081	0.098
5% ethanol	0.070	0.081	0.096	0.071	0.063	0.075	0.091
1% methanol	0.113	0.148	0.168	0.201	0.172	0.141	0.123
2% methanol	0.110	0.139	0.155	0.185	0.129	0.145	0.119
5% methanol	0.108	0.144	0.146	0.143	0.140	0.142	0.131

Consequently, we can exclude isopropanol from the list of organic extractants useful for anthocyanins extraction (Figure 2).

While extraction with methyl alcohol in the presence of 1% hydrochloric acid, the peak of anthocyanin concentration was observed after 4 h of maceration. Further on anthocyanins became discolored. In 1% ethanol, the concentration of anthocyanins continued to increase slightly during the entire incubation time, however, the maximum optical density was two times lower than in samples with methyl alcohol as a solvent. An increase in the percentage ratio of hydrochloric acid did not show any significant differences; therefore, 1% ethyl and methyl alcohols can be considered as effective organic solvents for the extraction of anthocyanins from black rice powder.

According to the literature sources, the extraction of anthocyanins might be increased by maceration in a water bath or in an US-machine. In order to find out how such methods are more effective than maceration at room temperature, 2.5 g of the sample were poured into 25 mL of an organic extractant and placed under various conditions. After each hour, an aliquot of 10 microliters was diluted 100 times and the optical density of the solution was measured. The measurement results are presented below (Table 2).

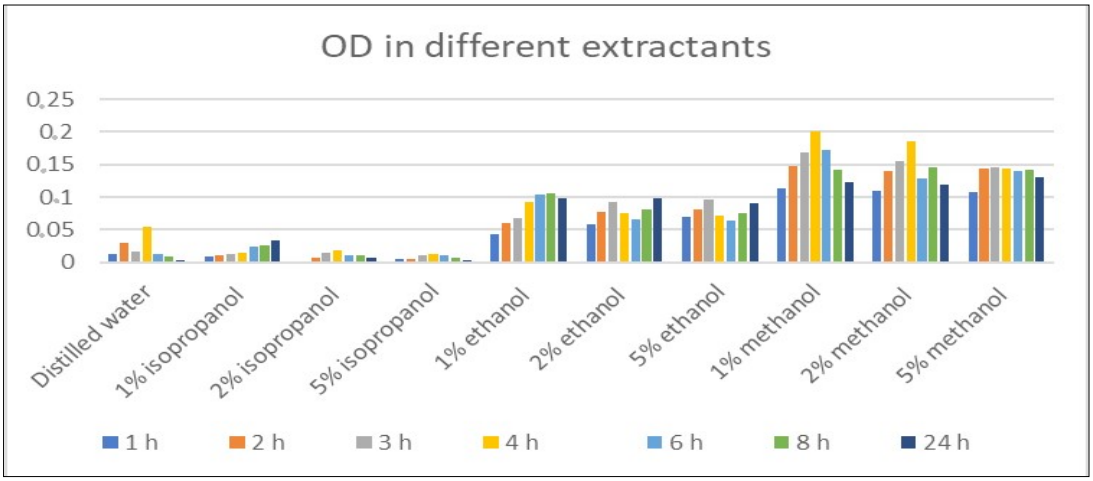

Figure 2 – Dependence of anthocyanins concentration on an extractant and time of incubation

Table 2 – The dependence of OD on different types of maceration at λ_{max}=538 nm

Conditions	1 h	2 h	3 h	4 h
Maceration at room temperature				
Ethanol	0.038	0.056	0.075	0.097
Methanol	0.139	0.147	0.165	0.185
Maceration at 75 °C in water bath				
Ethanol	0.089	0.131	0.142	0.159
Methanol	0.222	0.202	0.164	0.183
Maceration in US-machine				
Ethanol	0.195	0.156	0.122	0.117
Methanol	0.201	0.193	0.180	0.195
Maceration in both water bath and US-machine				
Ethanol	0.173	0.188	0.178	0.195
Methanol	0.192	0.178	0.189	0.183

Therefore, the optimal conditions for maceration were revealed. Figure 3 shows that the best results were obtained after 1 h maceration in the water-bath in the presence of methanol, and after 1 h maceration in the US-machine in the presence of ethanol. The usage of methyl alcohol instead of ethanol and placing the solution in a water bath for one hour allowed to increase the yield of anthocyanins by more than two times in comparison with ethanol.

A pH-spectrophotometry method was then used to calculate the concentration of anthocyanins in three samples. The total weight of anthocyanins per 100 g of raw mass is equal to 167 mg (Table 3).

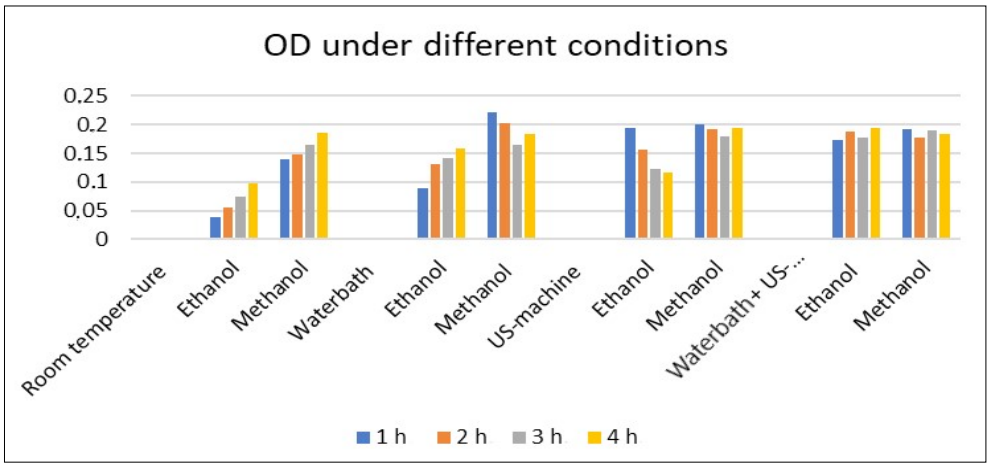

Figure 3 – The dependence of anthocyanins concentration on types of maceration

Table 3 – The total weight of anthocyanins per 100 g of raw weight

Source	pH=1.0	pH=4.5
Rice	510nm=0.030	510nm=0.015
	700nm=0.003	700nm=0.010
Note: C=167 mg/100g.		

The data obtained is confirmed with previous investigations.

Caudal fin regeneration. 264 fishes were selected randomly from the pool without any sign of diseases, physical nor anatomical abnormalities. Their length varied from 1.3 to 3.7 cm (Figure 5). The fishes were divided into two cohorts – control and experimental in equal proportions.

To avoid any mistakes and inaccuracies in the further calculations, before the experiment we measured TL and LCF (TL – total length; from the rostrum to the end of the longest fin ray, and LCF – length of caudal fin; the length of caudal rays) and processed the obtained data via T-test. It has been showed, that t_{emp} =0.8589 for TL< t_{crit} =1.972 (p=0.05, df=262), so, it can be stated, that there are not any differences in total body length for both groups. The same results were obtained for length of caudal fin. According to the calculations, t_{emp} =0.7817 for LCF< t_{crit} =1.972 (p=0.05, df=262, which proves that the cohorts of the zebrafish are homogeneous.

Table 4 – The results of TL and LCF measurements in control and experimental cohort right after the procedure of caudal fin elimination

	$TL_{control}$	$TL_{experimental}$	$LCF_{control}$	$LCF_{experimental}$
$\bar{x}$	2.76	2.75	0.56	0.59
m ($\sigma/\sqrt{(n)}$)	0.004	0.006	0.0217	0.0247
t_{emp}	0.8589		0.7817	
df (T)	262		262	
$t_{crit,}$ p = 0.05	1.972		1.972	

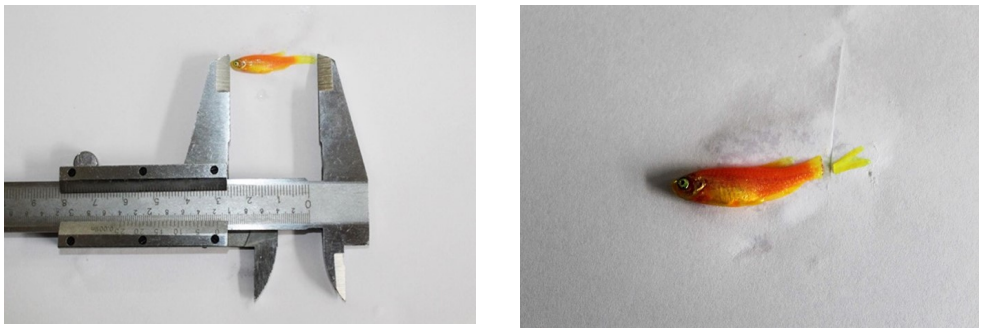

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Figure 4 – *Danio rerio* species. Note: A– before elimination (x10) and B– right after (x20)

Via microscope Motic and Motic Images Plus 3.0 software the pictures of fishes right after amputation were taken (Figure 5). As we can see on the picture, no veins nor arteries, neither vertebras were harmed. All fishes survived the procedure of caudal fin elimination.

Right after amputation fishes were transferred to a temporary tank, where visual assessment of their behavior was performed. Amputees demonstrated weak reaction on light (both natural and artificial), ignored food and preferred staying at the bottom, whereas non-amputees swam actively, reacted on tapping and consumed food. Such type of behavior which was detected in the amputees signals about stress, normal for animals deprived of limbs or other structures. 1 h later all fishes successfully passed the tests and had no differences in comparison with fishes with caudal fin. These observations were repeated several times to insure that stress had short-time effect and did not result in any severe consequences for fishes´ health as chronic stress could do.

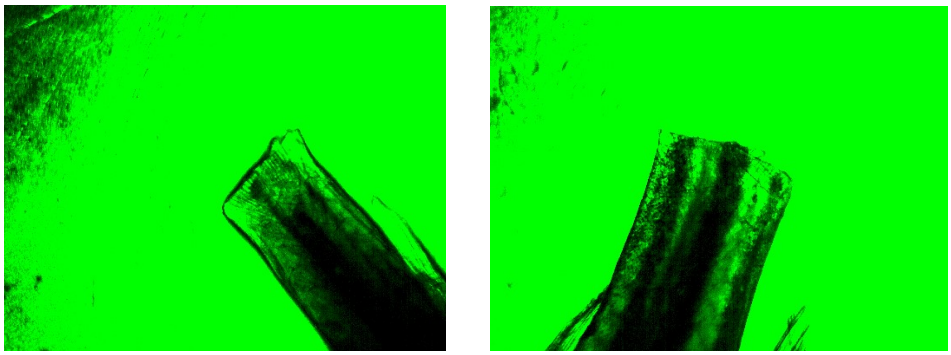

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Figure 5– Caudal fins of fishes right after amputation.

Note: A– control (x100); B– experiment (x100)

Within 3 weeks since the experiment was started:

– the control group was fed by a mix of commercial and natural food;

– an experimental group consumed the same mix soaked in anthocyanins.

Two days after the start, in both tankers dead representatives were detected. Probably, traumas, obtained during catching and measuring, temperature fluctuations, bad aeration, bacterial or fungal invasion etc. could provoke death of fishes. However, there were not any outbreaks of mortality, whereas in the experimental group fishes continued dying without any visible causes.

In 7 days the secondary measurements were done. The results are presented in table (Table 5).

As we can see one week later, there is no significant difference not only in length (TL and LCF both), but also in weight. In the control group fishes were gaining mass and growing, in the experimental the processes of grow and development were slowed down (Figures 6-7).

Table 5 – The results of TL and LCF measurements in control and experimental cohorts 7 days after the procedure of caudal fin elimination

	$TL_{control}$	$TL_{experimental}$	$LCF_{control}$	$LCF_{experimental}$
$\bar{x}$	2.463	2.345	0.21	0.19
$m (\sigma/\sqrt{(n)})$	0.028	0.033	0.0083	0.0077
t_{emp}	2.694		2.382	
df (T)	241		241	
$t_{crit,}$ p = 0.05	1.972		1.972	

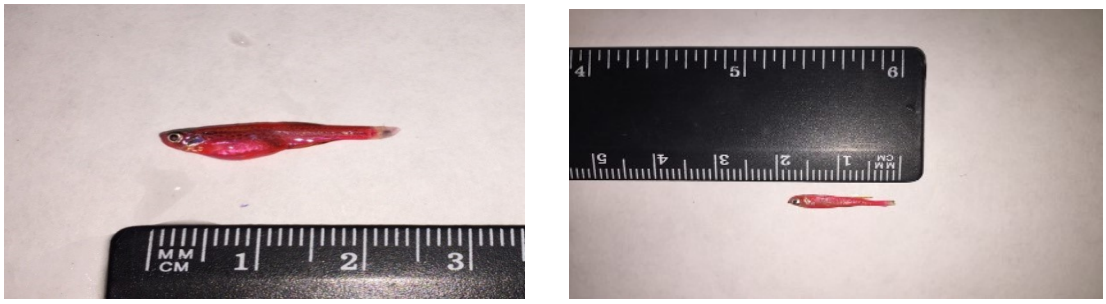

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Figure 6 – The process of caudal fin regeneration with eye vision. Note: A–control (x10); B–experiment (x10)

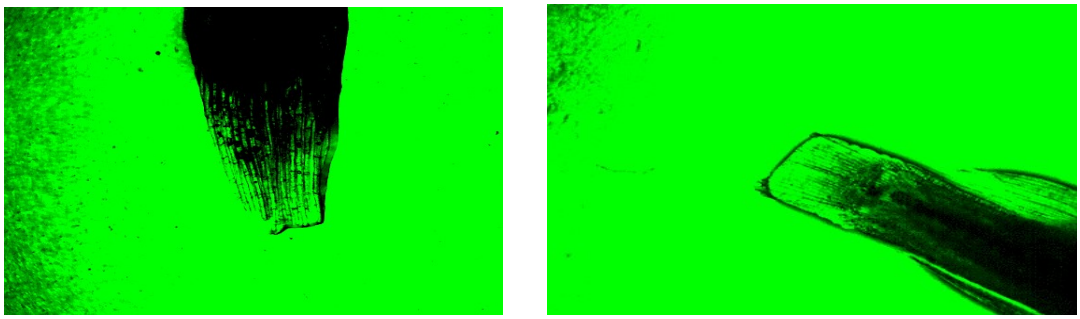

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Figure 7 – The process of caudal fin regeneration. Note: A - control (x100); B - experiment (x100)

In parallel with weekly measurements, we also performed visual evaluation of physical conditions and behavioral patterns of fishes. One of the most representative parameters is speed of food consumption. In the control subpopulation it was equal to 5 min (100% of food, no wastes), in the experimental was 11 min (80%). Physical activity also decreased, all fishes were passive, did not demonstrate research behavior, schooled and did not react on any stressors, like light and sound. The control ones actively swam in the tank in all directions, searched and reacted on knocking on the glass in a normal way. These behavioral differences proved again, that anthocyanins impacted not only grow processes, but also on general health and living conditions.

Two weeks later another series of measurements was done (Table 6).

Table 6 – The results of TL and LCF measurements in control and experimental cohorts 14 days after the procedure of caudal fin elimination

	$TL_{control}$	$TL_{experimental}$	$LCF_{control}$	$LCF_{experimental}$
$\bar{x}$	2.545	2.414	0.3	0.27
m ($\sigma/\sqrt{(n)}$)	0.0309	0.0428	0.0092	0.0090
t_{emp}	2.476		2.36	
df (T)	189		189	
$t_{crit,}$ p = 0.05	1.973		1.973	

As t_{epm}=2.476 for TL>t_{crit} =1.973 (p=0.05, df=189), ~~we accepted~~ $H_{(A)}$. It means, that two set of fishes are different in length. The same distinction can be observed in the length of caudal fin. t_{epm}=2.36 for LCF>t_{crit} =1.973 (p=0.05, df=189), so, in the control group the process of regeneration went better and faster in contrast to the experimental group.

Microscopic pictures allowed us to see the process of caudal fin restoration more detailed. On the left normally regenerating fin can be observed, fin rays have different length to form heterocercal lobes. On the right all fins are identical, skin fold is invisible, lobes cannot be detected (Figure 9). These morphological changes show that the process of regeneration has been interrupted.

Three weeks later new 18 dead experimental fishes were detected. Those fishes, that were still alive, did not show interest to food, interactions, stopped swimming and spent time lying on the bottom of the tank.

The third measurements revealed that the length of body in the control group significantly increased in comparison with the experimental group. Also, we can see the positive correlation between body length and caudal fin length.

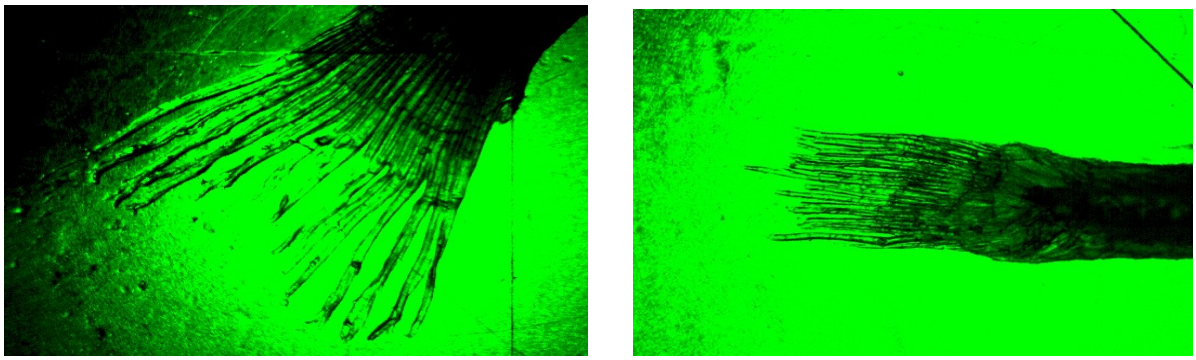

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Figure 8 – The process of caudal fin regeneration. Note: A- control (x100); B- experiment (x100)

Table 7 – The results of TL and LCF measurements in control and experimental cohorts 21 days after the procedure of caudal fin elimination

	$TL_{control}$	$TL_{experiment}$	$LCF_{control}$	$LCF_{experiment}$
$\overline{x}$	2.647	2.311	0.42	0.34
m ($\sigma/\sqrt{(n)}$)	0.013	0.021	0.027	0.031
t_{emp}	5.65		2.0	
df (T)	157		157	
$t_{crit,}$ p = 0.05	1.977		1.977	

Obviously, that anthocyanins acted as an agent, inhibiting the processes of growth and regeneration. According to literature sources, anthocyanins are considered as a substance with antitumor effect, suppressing some type of cancer, as colorectal adenoma. Anthocyanins regulate cell proliferation and mechanism of apoptosis. However, high concentrations of anthocyanins can stop not only cancer cells, but healthy and normal ones, abolishing mitosis and essential renewal of tissues and organs.

The main organ which is responsible for effective digestion is intestine. From the inside it is covered by non-keratinized epithelium, constantly damaged as a result of mechanical impact, and thus regularly replaced by new cells. The stop of cell division and self-renewal causes perforation of intestine, inner bleeding, deterioration of normal digestion, losing weight, weakening of immune response, and, at the end, death.

Based on the obtained results, we can say, that high concentration of anthocyanins in food, consumed day by day (15% and more from total mass of food) led to failure both in amitotic and mitotic cycles and provoked weakness, growth and regeneration suppression.

In the picture it has been shown that the caudal fin in the experimental group was not only shorter than in the control, but differed from it morphologically. We can see homocercalus fin with visially distinguishable lobes. In the control fin rays are homogeneous, no signs of tumor or atypical structures have been detected. Regeneration operates correctly. In the experimental group the length of fin rays different, no lobes can be seen, skin fold is rigid, what did not allow to straighten caudal fin fully. Food containing highy concentrated anthocyanins negatively influenced the processes of cell division. So, mechanisms of proliferation and regeneration were stopped prematurely, what resulted in pathological changes in the structure of caudal fin.

As some authors reported, high concentration of anthocyanins might induce apoptosis in different somatic cells in humans and mice [14]. Anthocyanins can block cell division at different stages of mitosis, because cyanidin glycosides accumulate ROS and have cytotoxic effect on tumor cells [15]. Also, it has been found, that anthocyanins increase the concentration of caspase-3, -8 and -9, and initiate degradation of poly(ADP-ribose) polymerase [16].

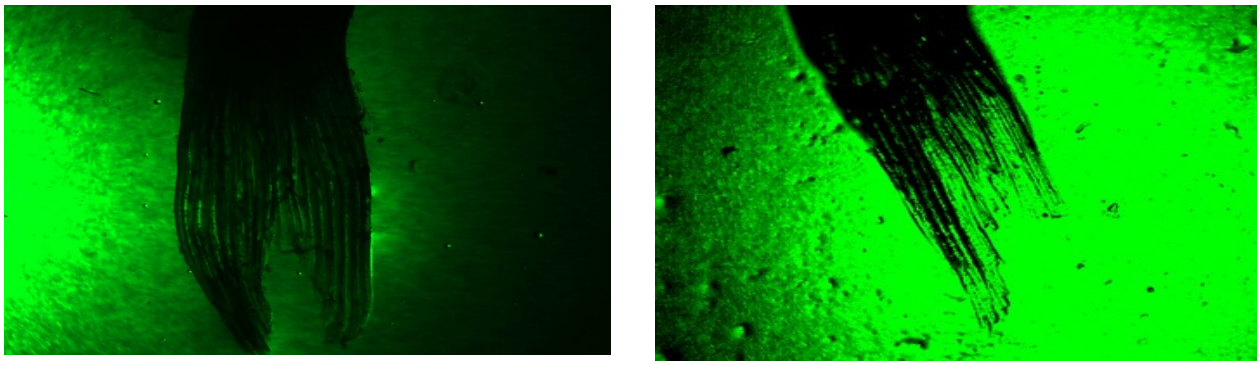

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Figure 9 – Caudal fin of Danio rerio in a group. Note: A–control (x100); B–experimental (x100)

Conclusion

Anthocyanins are natural polyphenol compounds, which stimulate vivid colors to numerous vegetables, fruits and cereals. These pigments range in color from orange-red to (dark) blue-violet and could serve as natural colorants to replace artificial additives. There is a tremendous demand from consumers to have fewer artificial compounds in their foods. Despite food supplements based on anthocyanins are widely spread in the world, in fact, there are not strict evidences that they have any positive effects on human health. According to the obtained results it was defined that in high concentrations anthocyanins decrease the process of regeneration, cell proliferation and tissue formation in *Danio rerio*. In obedience to literature sources, high concentrations are associated with increase of caspase level in cells and induced caspase-dependent degradation. Major caspase cascades leading to apoptosis since they are able to slay not only tumor cell, also healthy ones. Effector caspases are responsible for initiating the hallmarks of the degradation phase of apoptosis, including DNA fragmentation, cell shrinkage and membrane blebbing. These investigations will be prolonged to unveil the inner mechanisms, provoked such critical changes in caudal fin regeneration or outer physical appearance alterations.

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