Testing the Effect of Nutrients for The Personalized Nutrition with The *Danio Rerio* Fish (On the Example of The Nanodessert Nutritional Fermented Dairy Product)

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Abstract *The effect of the new Nanodessert nutritional biological product (the Nn product) has been studied on the juvenile Danio rerio laboratory fish, which were used for detecting the stimulating properties of the Nn product on the two important biological functions: the erythropoiesis, and the regeneration of the lost organs. As a result of the morphological and fluorescent histochemical analysis, it has been found that the most intensive regeneration of the fin lobe occurs on day 8 of the experiment. At this time, the growth of the cut-off upper lobes of the caudal fins of the fish that receive the Nn product with the feed is by 42 % greater than that of the regenerate of the fish that do not receive the Nn product. The histochemical studies have shown that in the regenerating caudal fin blastemas under the influence of the substances contained in the Nn product, the amount of RNA also increases, which contributes to increasing the protein biosynthesis and accelerates the restoration of the lost parts of the organs.*

Keywords — *Nanodessert nutritional product, zebrafish, stimulation, erythropoiesis, regeneration, RNA.*

I. INTRODUCTION

The effect of the Nanodessert nutritional product (the Nn product) on the hematopoietic and regenerative processes upon its addition to the feed for the *Danio rerio* aquarium fish was studied using the cytochemical methods. It was shown on the number of young erythrocytes in the peripheral blood of the fish and the regeneration of the caudal fin upper lobes that adding the Nn product to the Esturgeon granulated feed acted as a stimulating factor of the blood formation. The *Danio rerio* fish are currently widely used in the laboratory studies by oncologists and biologists for identifying carcinogenic and anticarcinogenic compounds, and substances with stimulating properties [1, 2]. The authors also suggested using these experimental objects for testing the effect of the nutrients in personalized nutrition. Many genes of the zebrafish are similar to those of humans and other mammals, including the p53 suppressor gene, which is responsible for the elimination of cancer cells [3].

Studying the cytodifferentiation of erythrocytes in the peripheral blood allowed assessing the presence of the stimulating or inhibitory property in the food products by the the young erythrocytes occurrence rate in the peripheral blood, in which the DNA morphology and structure were different from those of the erythrocytes that had completed cell differentiation. An increase in the number of young erythrocytes in the blood showed that the mitotic activity in the cells of the erythropoiesis series was stimulated with the Nn product.

In addition to revealing the stimulating effect on the erythropoiesis, the authors studied the effect of the Nn product on the regeneration, when the rate of recovery of the cut-off upper lobes of the zebrafish caudal fins was used for assessing the stimulating effect of the studied product. The regeneration was accompanied by the dedifferentiation of the cells, the growth of the blastemas, and subsequent differentiation of the cells. In most vertebrates, including humans, these processes are similar. Therefore, the data obtained for the fish allow stating that the Nn product has the property of stimulating the regeneration of tissues and organs in other species of vertebrate animals as well, including humans.

This study was aimed at identifying the presence or absence of stimulating properties in the Nn product on important biological processes, such as erythropoiesis and the regeneration of organs and tissues, which had been found in the *Danio rerio* laboratory fish.

II. MATERIALS AND METHODS

The Nn product was tested for its ability to stimulate the erythropoiesis (and, consequently, the mitotic activity) and the regeneration of parts of the lost organs through the use of the morpho-cytochemical analysis of the erythrocytes nuclei and studying the regeneration rate of the upper lobes of

the caudal fins in the juvenile Danio rerio fish. Ten two-centimeter-long fish at the age of two months were placed into a plastic aquarium with settled tap water. Each aquarium contained two liters of water. A total of five aquariums with the zebrafish were installed. The first aquarium contained the intact fish fed with the Esturgeon French feed with the grain size of 200 – 300 microns (reference 1). The next aquarium contained the fish with the upper lobes of cut-off caudal fins, which received the feed without the Nn product (reference 2). The remaining three aquariums with the zebrafish were used for the experiment where the fish received the feed with the Nn product added; the upper lobes of their caudal fins had been cut off. Before cutting off the lobes of the fins, the fish had been adapted to the new conditions for a week. Feeding the fish with the addition of the Nn product in the experimental aquariums was started immediately after the fish had been placed in the aquariums. The upper lobes of the caudal fins were cut off with a microscalpel one week after the fish had been placed into the experimental aquariums under the monitoring with an MBS-10 binocular microscope. The lower lobes were used for reference (the upper and the lower lobes of the fins are approximately equal in the area and morphology) to determine the size to which the cut-off lobes of the fins should regenerate.

In total, including the adaptation to the keeping conditions, the fish received the feed with the addition of the Nn product (15 days), and the regeneration process was studied for eight days (the period of the most intensive regeneration of the fins) after the upper lobes of the caudal fins had been cut off.

Since zebrafish don't take granules without feed, the granules of the Nn product were pressed into the feed in a porcelain mortar using a pestle without destroying the granules. After that, it became possible to give calculated dosages of the product to the fish. It was found by calculation and proven experimentally that a single juvenile fish with the weight of 300 mg should receive 15 mg of the granulated feed and 3 mg of the Nn product daily. The experiment lasted for 15 days, during which the effect of the Nn product on the erythropoiesis processes in the blood-forming organs of the fish was studied.

To study the cellular elements of the blood, crushed drop preparations of the live blood were prepared to allow two weeks from the start of the experiment to pass. The resulting preparations were used for cytochemical and morphological analysis of the erythrocytes nuclei using fluorescent microscopy.

For each analysis, blood from five fish was taken. The blood was taken by cutting the tail stem, which allowed obtaining a small drop of blood on the glass slide, onto which a solution of acridine orange was placed, followed by a glass cover, and after five minutes, which were required for the dye to act, the preparation was ready for studying. Thus, there was no need for a blood smear (the method used in the hematological analysis), but the crushed drop preparation was prepared, which is widely used in microscopy for studying living cells. The intravital blood study was performed using a Biomed 3L luminescent microscope, which made it possible to identify the morphology and cytochemistry of erythrocytes and to determine the degree of their cytodifferentiation by the shape of the nucleus.

For the intravital detection of DNA in the erythrocytes nuclei, the authors used the acridine orange luminescent dye. A fresh drop of the fish blood was dyed following the generally adopted method (Pierce, 1963) (according to Bertalanffy and Bickins) with a 0.1 % solution of orange acridine diluted with nine parts of the Krebs-Ringer buffered solution.

The main feature of young erythrocytes that had not yet completed their cytodifferentiation, but were already in the peripheral blood, was the roundness of the nucleus and the presence of weak orange or yellow glow, which indicated the presence of RNA synthesis in them. At the same time, oval nuclei and green fluorescence were observed in old erythrocytes, which indicated the termination of the genetic information transcription upon the DNA molecules transition to the two-chain stage.

According to the generally adopted guidelines for microscopic analysis of erythrocytes [4, 5], 2,000 erythrocytes were accounted for in the reference and each experimental group, and the frequency of young cells occurrence relative to the fully differentiated cells was considered.

To study the effects of the Nn product on the regeneration process in all the fish in reference 2 and in the experimental groups, the upper lobes of the homocercal fins were cut off seven days after placing the fish into the aquariums. Next, the growth of the upper lobe blastemas was observed under an MBS-10 stereoscopic microscope and photographed on days 3, 6 and 8.

To assess the regeneration of the upper lobes of the caudal fins, the ratio of the areas of the regenerating and the lower lobes of the fins was determined. This allowed obtaining a more objective response even in the fish with various growth rates. To determine the area of the blastemas and the lower lobes of the fins, a mesh was superimposed on the computer image of the zebrafish caudal fin, and the total area of the blastemas and the area of the lower lobes were calculated from the mesh area (Figure 1). The results were statistically processed according to the Student's t-test.

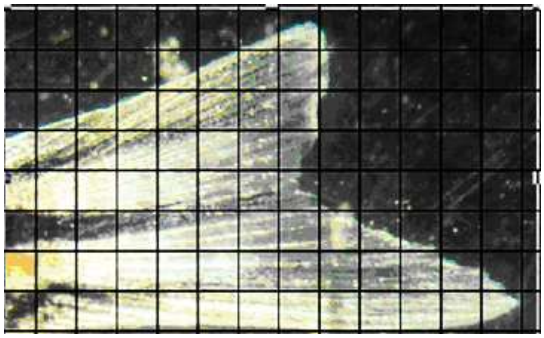
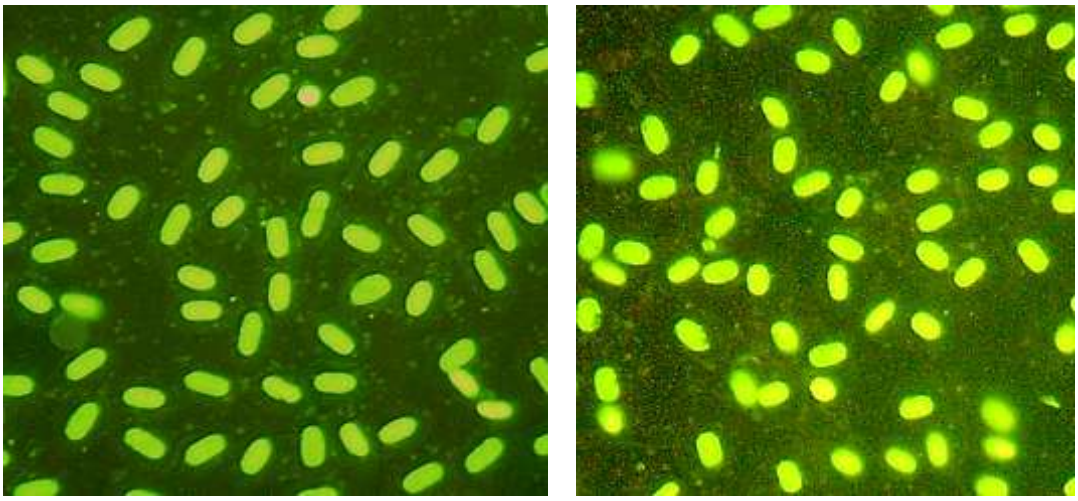


Fig. 1: Superimposing a mesh for calculating the ratio of the areas of the regenerating and the lower lobes of the zebrafish caudal fins (magnification of 10 x 12.5.

III. RESULTS

A. Erythropoiesis

To identify the stimulation of the blood cell division in the zebrafish in the case of erythropoiesis under the effect of the Nn product, the young erythrocytes occurrence rate was determined, compared to that of the erythrocytes that had completed cytodifferentiation. Figure 2 below shows two random spots on the crushed drop preparation in the reference and the experimental groups. In the fish that received the Nn product for 15 days, erythropoiesis was more intensive than in the reference. The young to fully differentiated erythrocytes ratio was 1:55 in the reference, and 2:34 in the experimental groups.



a b

Fig. 2: The picture of the peripheral blood of the zebrafish in the reference (a) and the experimental groups (b). View in a luminescent microscope (the acridine orange dye) (magnification of 40 x 15). Erythrocyte nuclei are visible (DNA turns green).
a – reference – fish feeding without the Nn product (1 reticulocyte)
b – experiment – fish feeding with the Nn product (3 reticulocytes are noted).

Table 1 below shows the young erythrocytes occurrence rate in the blood of the fish, compared to that of the fully differentiated erythrocytes, which provides the possibility to assess the erythropoiesis stimulation under the effect of the Nn product.

Table 1: The young erythrocytes occurrence rate, compared to that of the erythrocytes that had completed cytodifferentiation in the blood of the zebrafish in the reference, and the fish that received the Nn product as an additive to the feed

Indicators	Reference 1	Reference 2	Experiment 1 with the Nn product	Experiment 2 with the Nn product	Experiment 3 with the Nn product	Average with the Nn product
Young erythrocytes (occurrence rate ‰)	1.8	1.5	5.8	5.5	4.8	5.4
Stimulation of erythropoiesis (%)	100	100	322	305	266	297

Thus, feeding the experimental fish with the addition of the Nn product to the feed intensifies the erythropoiesis almost 3.0 times. It may be indirectly assumed that the mitotic activity of the cellular elements in the case of erythropoiesis increases by the same value. The comparative analysis of the erythropoiesis in reference 1 and reference 2 showed that cutting off the lobes of the caudal fins during the experiment partially reduced the number of young

erythrocytes, but this difference was not statistically veracious.

From the experiments performed, a conclusion may be drawn that the Nn product increases about 3 times the mitotic activity during the erythrocytes maturation and stimulates erythropoiesis in the zebrafish.

B. Regeneration

The rate of the regeneration of the caudal fins in the zebrafish was determined in the experimental and the reference groups by the ratio of the areas of the upper regenerating and the intact lower lobes of the homocercal fins. Figure 3(a) shows the regeneration of the caudal fin lobe in the zebrafish on days 3, 6, and 8 after cutting off the upper lobes. The previous experiments had shown that the regeneration of the upper lobes of the caudal fins in the juvenile Danio fish had been the most intensive until day 8, and had ended by day 12. For this reason, the authors only studied the most intensive period of the regeneration of the upper fin lobes until day 8.

Figure 3(b) shows the regeneration of the caudal fins in the fish that received the Nn product. The stimulating role of the Nn product is visible, and is further confirmed by the calculation of the blastemas to the lower lobes areas ratio

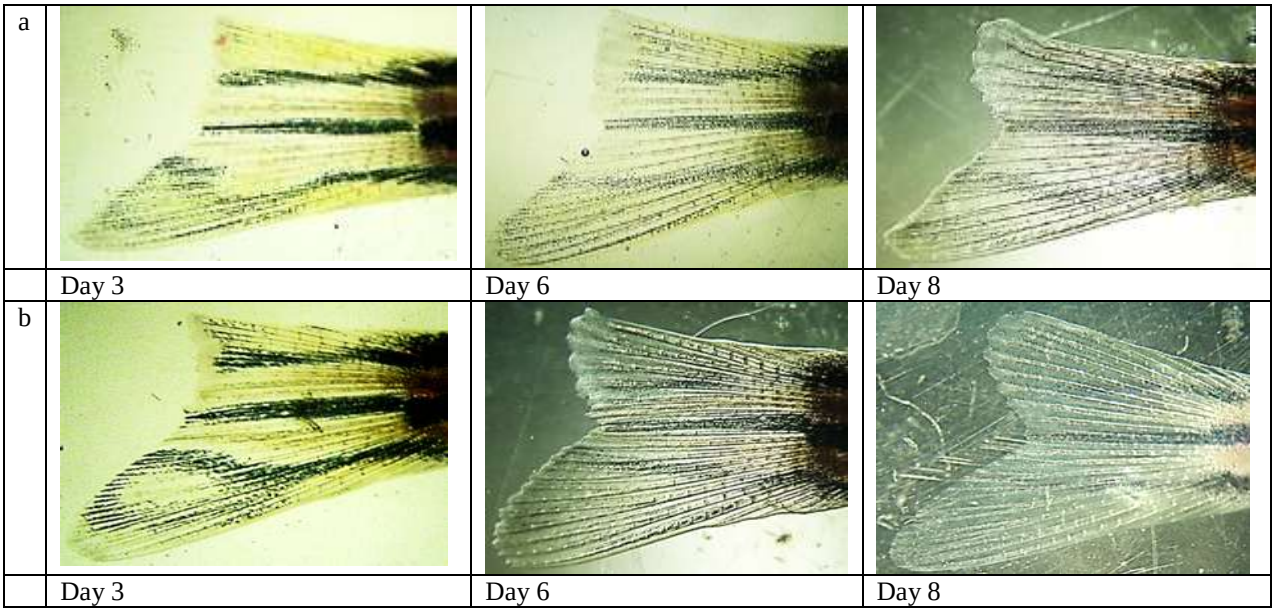


Fig. 3: The regeneration of the upper lobes of the caudal fins in the zebrafish (a: without the Nn product in the reference, and b: with the Nn product added to the feed in the experimental groups).

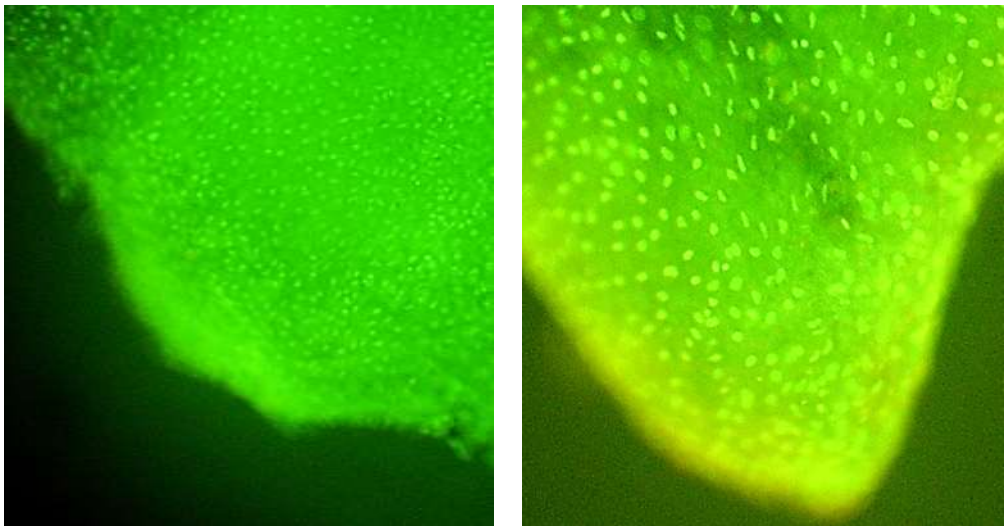
Figure 4(a, b) shows that the regeneration of the upper lobes of the caudal fins in the fish that received the Nn product was about 40 % faster than in the reference. More accurate calculation of the caudal fin upper lobes regeneration rate was made using the ratio of the areas of the blastemas and the bottom parts of the fin lobes (the results are shown in Table 2).

Table 2: The ratio of the areas of the blastemas and the bottom parts of the fin lobes during the caudal fin regeneration in the zebrafish

Days of regeneration	Day 3	Day 6	Day 8
With the Nn product	0.065* ± 0.02	0.31* ± 0.04	0.77* ± 0.06
Without the Nn product	0.038 ± 0.03	0.14 ± 0.04	0.31 ± 0.02

Thus, the most intensive regeneration ended on day 8; it was characterized by the regenerate growing by 46 % faster than in the reference fish that did not receive the Nn product with the feed. The beginning of the regeneration of the caudal fin upper lobes in the zebrafish was slower; however, the regeneration rate in the presence of the Nn product still tended to increase.

To assess the effect of the product at the histochemical level, the content of nucleic acids was determined in the blastemas of the caudal fin upper lobes of the zebrafish using the acridine orange fluorochrome. An intravital luminescent study of the regenerating fin fragments was performed with staining the preparation with acridine orange. The preparations were made from the blastemas of the fins of the fish in the reference, in which the regeneration passed without the product, and of the fish that received the Nn product. The nucleic acids were detected in the regenerate of the blastemas on day 8, at the end of the experiment. Table 2 shows that it was the most active period of the regeneration of the caudal fin lobes. Figure 4 shows the preparations of the edge of the blastemas with slightly differentiated cells of the regenerating fins on day 8 after cutting off the upper lobe (a — reference 2 and b — experiment) in the field of view of a luminescent microscope.



a b

Fig. 4: The edge of a blastema of a regenerating fin lobe of the zebrafish on day 8 of the experiment with dedifferentiated cells (luminescent microscope and the acridine orange dye) (magnification of 8 x 15).
a – Fluorescence of the nucleic acids (mainly DNA) in the fin blastemas of the fish that did not receive the Nn product.
b – The blastemas of the zebrafish caudal fins regenerate with the fluorescence that indicates a high content of DNA (green glow) and RNA (orange glow), typical of the fish that received the Nn product.

The histochemical analysis of the preparations in Figure 4(a) showed that without adding the Nn product to the fish feed, DNA was identified in the blastemas (green fluorescence), and the tissues in the area of the cut in the fin lobes were activated. At the same time, RNA synthesis was suppressed. In the fish that received the Nn product with the feed, RNA synthesis in the blastemas was noted (Figure 4(b)). RNA was the most intensively detected in the peripheral parts of the blastemas (orange glow). Intensive RNA synthesis in the blastemas indicates the intensification of metabolism in the regenerate after adding the Nn product to the feed of the fish, compared to the reference. On the background of RNA synthesis, larger cell nuclei were detected, which also indicated an increase in the functional activity of the genetic apparatus during protein biosynthesis.

Since the fluorescent method detected all RNA types, and they were difficult to differentiate, it was possible that among these nucleic acids in the blastemas, microRNA might be present, which actively participated in the cytodifferentiation after the mitotic activity in the tissues had ended [6-9].

Thus, the Nn product acts as a stimulant for the regeneration of tissues and organs. The rate of the lost parts of the organs regeneration was not constant, and in certain periods varied from 12 to 46 %.

These results were also confirmed by the results of the histochemical studies with the use of fluorescent microscopy. The Nn product accelerated RNA synthesis in the blastemas, and, consequently, subsequent protein biosynthesis, which had a stimulating effect on the regeneration processes.

IV. CONCLUSIONS

The new active biological Nn product has been studied on the juvenile *Danio rerio* laboratory fish, which have been used for detecting the stimulating properties of the Nn product on the following important biological functions: erythropoiesis and the regeneration of the lost organs. The fish have been given the feed with 16 % of the Nn product added. In the first part of the experiment, it has been found that feeding the fish with adding the Nn product increases about three times the young erythrocytes occurrence rate in the peripheral blood of the zebrafish, compared to old erythrocytes, which indicates stimulation of erythropoiesis. In the second part of the experiment, the presence of a stimulating property of the Nn product capable of accelerating the regeneration of the cut-off upper lobes of the caudal fins in the zebrafish was studied. As a result of the morphological and fluorescent histochemical analysis, it has been found that the most intensive regeneration of the fin lobes occurs on day 8 of the experiment. At this time, the growth of the cut-off upper lobes of the caudal fins in the fish that receive the Nn product with the feed is by 42 % greater than the growth of the regenerate in the fish that did not receive the Nn product. The histochemical studies have shown that in the regenerating blastemas of the caudal fins under the influence of the substances contained in the Nn product, the amount of RNA also increases, which contributes to increasing the protein biosynthesis and accelerates the restoration of the lost parts of the organs. Thus, the studied biologically active Nn product acts as a stimulant for the

regeneration of tissues and organs. The stimulating properties of the Nn product found in the experiment with the fish have an effect on the universal biological mechanisms of regeneration and erythropoiesis; therefore, it is possible to transpose the data obtained to other species of the vertebrate animals, including humans. The identified properties of the studied product allow concluding that the Nn product will be widely used in personalized nutrition and biology. The work also shows that the *Danio rerio* laboratory fish may be successfully used for screening individual nutrients to identify their properties that the man needs in a personalized diet.

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