Experimental paper

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Effect of dietary administration of kynurenic acid on the activity of splenocytes of the rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Kynurenic acid (KYNA) is a metabolite of tryptophan that an organism produces constitutively along the kynurenic pathway. This substance, known as an endogenous neuroprotectant, also demonstrates some immunotropic properties. The purpose of this research has been to determine the influence of kynurenic acid administered in feed on the proliferative activity of lymphocytes (MTT assay) and activity of phagocytic cells (RBA and PKA assays) isolated from the spleen of a rainbow trout. The experiment was conducted on 100 fish, divided into 4 groups: control, not receiving kynurenic acid, and three treatment groups which were given KYNA in feed, in the doses 2.5, 25 or 250 mg/kg of feed for 7, 14 or 28 days. The strongest effect of KYNA was observed after the shortest time of its administration. It manifested itself as a depressed proliferative response of T and B lymphocytes in the group given the highest dose of KYNA and, irrespective of the dosage, as an elevated activity of phagocytic cells. The effect weakened as the administration of KYNA continued. After 28 days of administration, the increased respiratory burst activity of phagocytic cells remained only in the group receiving the highest dose of KYNA. Another, rather unexpected effect observed on day 28 was the stimulated proliferation of T lymphocytes in the treatment with the lowest KYNA dose. The results confirm the immunotropic characteristics of kynurenic acid, as of today not investigated on a model of poikilothermic animals, which encourages further investigations into possible applications of the acid in fish therapy.

Key words: *kynurenic acid, rainbow trout, splenocytes, proliferative response, phagocytic cells activity.*

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Introduction

Tryptophan is one of the essential amino acids which human or animal bodies are unable to synthesize *de novo*, hence it must be supplied in adequate quantities with food. However, just about 30% of the dietary pool of tryptophan is incorporated into proteins, while the remaining amount is metabolized via various pathways to different, bioactive end products. The principal metabolic pathway for tryptophan is the kynurenic pathway, which performs regulatory functions in the nervous and immune systems [1]. One of the metabolites of this pathway is kynurenic acid (KYNA), an organic hydroxy acid, an antagonist of ionotropic glutamate receptors (NDMA) and α-7 nicotinic acetylcholine receptors (nACh) in the nervous tissue, known mainly as an endogenous neuroprotectant. For some time, it has been known that KYNA is also able to activate GPR35 receptors, present on the surface of different populations of cells in the immune system. A strong expression of these receptors has been observed on monocytes, T cells, neutrophils and dendritic cells; a weaker expression was noticed on B cells, eosinophils, basophils and NKT cells. The immunotropic properties of KYNA described so far, and originating from its reaction with GPR35 receptors, are predominantly associated with the anti-inflammatory action [2-5]. Apart from immune cells, GPR35 receptors are also present on the surface of enterocytes in intestinal crypts, which may imply some involvement of KYNA in the local immunologic response in the digestive tract and in the functions performed by enterocytes. Until today, it has been confirmed that kynurenic acid affects the mitotic activity and differentiation of the epithelium of the digestive tract and that it can protect against ulceration or other damage of mucus membranes in the digestive system or even – to some extent – against intoxication [6]. It is also known that KYNA is effectively absorbed in the digestive

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tract [7], which, in combination with its ability to affect the immune system, stimulates research on using KYNA as a natural immunomodulator in prophylaxis and therapy of infectious and inflammatory diseases.

Among all reared animals, fish are one of the major targets of immunomodulation owing to the specific nature of their immunologic system. The evolutionary status of fish and their poikilothermic bodies restrict the potential of combating pathogens by specific immunity, thus ensuring the dominance of the non-specific response, which is more immediate and relatively independent from the temperature [8]. This is the reason why the preparations enhancing functions of the immune system of fish are popular in aquaculture, especially substances from natural sources, which – owing to their high biocompatibility and biodegradability – are safe for the natural environment and the health of future consumers.

The purpose of this study has been to determine the influence of exogenous kynurenic acid, administered in different doses and for different time lengths in feed, on the proliferative response of lymphocytes and on functions of phagocytic cells isolated from the spleen of rainbow trout. This article presents the first attempt at determination of the immunotropic properties of KYNA on a fish model.

Material and methods

Fish

The experiment was conducted on 100 rainbow trout individuals with the average body weight of 150 g. The fish were maintained in the aerated freshwater tanks at 17°C, with a 12 h light/12 h dark period. Followed by 7 days of acclimation the animals were randomly divided into 4 equal groups: the control, which was fed commercial feed without a supplement of kynurenic acid, and 3 treatment groups, which were given kynurenic acid (Sigma-Aldrich) added to feed in the following doses: 2.5, 25 and 250 mg/kg. In the first week of the experiment, the fish were given a ration of feed equal to 1% of the body weight a day; in the following weeks, the ration was increased to 2% of the body weight. The fish readily forage on the feed and the supplementary dose of KYNA was not observed to have affected the body weight gains, general condition of the fish or their behaviour. After 7, 14 and 28 days of the administration of KYNA in feed, 8 fish from each group were sacrificed. Circulating blood was collected from fish anaesthetized with 2% Propiscin (Żabieniec, Poland) diluted in water (1 ml/l) by caudal vein puncture, and the spleen was sampled after bleeding. The experiment has been approved by the Local Ethics Committee.

Isolation of leukocytes

Leukocytes for the tests were isolated from the fish spleen. The spleens were removed aseptically, and pressed through a 60-μm nylon mesh in RPMI-1640 medium with L-glutamin and sodium bicarbonate (Sigma-Aldrich). The splenocyte cell suspensions were placed on density gradients: Gradisol G (Aqua-Medica, Łódź, Poland) – in order to isolate phagocytic cells or Gradisol L (Aqua-Medica, Łódź, Poland) – in order to isolate lymphocytes, and then centrifuged at 400 g for 40 min at 4°C. The interface cells were collected and washed three times with the RPMI-1640 medium at 400 g for 5 min. Viability of isolated cells was evaluated by trypan blue exclusion and was determined to be greater than 95% in each case. Cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS, Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich), then dispensed into 96-well plates at a concentration of 2×10^6 cells/ml, cultured/incubated at 22°C and used for the following assays.

The proliferative response of lymphocytes – MTT assay

The mitogenic response of lymphocytes was determined using the MTT colorimetric assay [9]. Cells were suspended in RPMI-1640 growth medium containing mitogens – concanavalin A (ConA, Sigma-Aldrich) in concentration of 50 μg/ml as a T-cell mitogen or lipopolysaccharide from *Serratia marcescens* (LPS, Sigma-Aldrich) in concentration of 50 μg/ml as a B-cell mitogen and 100 μl of the suspension was added to each well of microtitre plates. The mixture was incubated for 96 h at 22°C. After incubation, 25 μl of the solution containing 7 mg/ml of MTT (3-[4, 5 dimethylthiazoly-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) in PBS were added and the plate was incubated for the next 4 h. The supernatant was removed and 100 μl of DMSO was added to each well. The optical density was measured at a wavelength of 570 nm with 640 nm as a reference wavelength. Samples obtained from each individual were tested in triplicate. The results of the proliferation assay were expressed as a stimulation index (SI), which was calculated by dividing the mean O.D. of stimulated cultures by the O.D. of the non-stimulated (control) cultures.

Respiratory burst activity (RBA) test

The metabolic activity of phagocytes was determined by the measurement of the intracellular respiratory burst activity after stimulation with PMA (phorbol myristate acetate, Sigma-Aldrich), as described by Chung and Secombes [10] with some modifications described by Chettri *et al.* [11]. 100 μl of cell suspension was added to each well of 96-well microtitre plates (Nunc, Denmark). After incubation for 2 h at 22°C, the cells were washed in RPMI 1640 medium to remove nonadherent cells and incubated for the next 24 h. Then, 100 μl of PMA (1 μg/ml) in 0.1% NBT (nitroblue tetrazolium, Sigma-Aldrich) solution in RPMI 1640 medium were added to each well. The mixture was incubated for 60 min at 22°C. After the removal of the medium from the cells, the reaction was stopped by the ad-

dition of absolute ethanol and then washed twice with 70% ethanol. The formazan produced in the cells was dissolved in 120 µl of 2M KOH and 140 µl of DMSO (dimethyl sulfoxide, POCh, Gliwice, Poland) and the optical density was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. Then, the indices of cells activity were calculated by dividing the mean O.D. of PMA stimulated cells by the O.D. of the non-stimulated cells. The results of the RBA assay were expressed as a percent of the control group phagocytes activity.

Potential killing activity (PKA) test

The technique presented by Rook *et al.* [12] was used to measure the potential killing activity of phagocytes. After removing nonadherent cells and the following 24 h incubation of cell, 100 µl of isolated cell suspension was mixed with 100 µl of 0.1% NBT solution in PBS (phosphate-buffered saline) (Biomed, Lublin, Poland) containing *Aeromonas hydrophila* (1×10^8 cells/ml) and incubated for 60 min at 22°C. After incubation the supernatant was removed from each well and adherent cells were fixed with absolute ethanol. 120 µl of 2M KOH and 140 µl of DMSO were added to each well and the plates were mixed. The amount of extracted reduced NBT was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. Then, the indices of cells activity were calculated by dividing the mean O.D. of bacteria stimulated cells by the O.D. of the non-stimulated cells. The results of the RBA assay were expressed as a percent of the control group phagocytes activity.

Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA). Bonferroni's post test was used to determine differences between groups. Statistical evaluation of results was performed using GraphPadPrism software package.

Results

Effect of kynurenic acid on the proliferative response of lymphocytes

After 7 days of the administration of the highest KYNA dose (250 mg/kg) in feed, a considerable decrease was observed in the proliferative response of both T ($p < 0.05$) and B cells ($p < 0.001$) relative to the values recorded in the control group. However, this effect did not persist over a longer period of KYNA administration.

Another statistically significant change, such as a higher T cell proliferation index ($p < 0.05$), was noted after the longest administration period (28 days) in the group given the lowest dose of KYNA (2.5 mg/kg). No significant differences in the above parameter against the control were recorded during the shorter periods of the administration of the said dose of kynurenic acid (Table 1).

The intermediate dose (25 mg/kg), irrespective of the duration of its administration, was not observed to have evoked significant changes in the proliferative activity of splenocytes (Table 1).

Effect of kynurenic acid on the activity of phagocytic cells

Seven-day administration of KYNA, regardless of its dose, significantly increased the activity of phagocytic cells compared with the control group, both in terms of the respiratory burst activity (the RBA assay) and the potential for intracellular killing of microorganisms (the

Table 1. Effect of dietary administration of kynurenic acid on the proliferative response of splenocytes of the rainbow trout (SI)

Time (d)	Mitogen (50 µg/ml)	Measures	Groups/dose of KYNA (mg/kg); *n* = 8			
			0 (control)	2.5	25	250
7	ConA	M	1.184	1.047	1.457	0.786*
		SD	0.167	0.112	0.507	0.098
	LPS	M	1.091	0.951	0.973	0.843***
		SD	0.169	0.133	0.086	0.114
14	ConA	M	1.217	1.084	1.107	1.104
		SD	0.163	0.105	0.141	0.137
	LPS	M	1.111	0.986	0.978	0.953
		SD	0.215	0.096	0.095	0.108
28	ConA	M	1.316	1.485*	1.342	1.179
		SD	0.111	0.135	0.156	0.089
	LPS	M	1.089	1.064	1.031	1.067
		SD	0.079	0.071	0.069	0.064

Table 2. Effect of dietary administration of kynurenic acid on the activity of phagocytic cells isolated from the spleen of the rainbow trout (% of activity of cells relative to the control)

Time (d)	Assay	Measures	Groups/dose of KYNA (mg/kg); *n* = 8			
			0 (control)	2.5	25	250
7	RBA	M	100	125.581*	132.674**	136.279**
		SD	22.093	22.209	18.255	18.837
	PKA	M	100	130.53**	133.525***	124.424*
		SD	19.009	19.7	10.944	14.631
14	RBA	M	100	99.114	105.315	105.216
		SD	14.764	24.212	12.401	10.433
	PKA	M	100	117.821	122.701**	123.723**
		SD	17.253	9.762	11.464	18.956
28	RBA	M	100	99.074	105.278	115.741**
		SD	9.907	7.778	14.074	7.221
	PKA	M	100	90.987	104.506	106.712
		SD	11.122	7.382	10.067	6.903

M – mean, SD – standard deviation
** – difference statistically significant in comparison to the control group (0) at p < 0.05*
*** – difference statistically significant in comparison to the control group (0) at p < 0.01*
**** – difference statistically significant in comparison to the control group (0) at p < 0.001*

PKA assay). The two higher doses of kynurenic acid (25 and 250 mg/kg) stimulated the activity of respiratory burst ($p < 0.01$) more strongly than the lowest dose ($p < 0.05$). In turn, the killing activity of splenocytes was more intense under the two lower doses (2.5 mg/ml – $p < 0.01$; 25 mg/ml – $p < 0.001$) compared to the highest one ($p < 0.05$) (Table 2).

As the time period of administering KYNA in feed grew longer, the activity of phagocytic cells was depressed, declining close to the level recorded in the control group. After 14-day-long administration of KYNA, the phagocytic activity was still elevated under the two higher doses of the acid 25 and 250 mg/kg ($p < 0.01$), whereas after 28 days, a more intense respiratory burst occurred only in the group given the highest dose of KYNA compared to the control group ($p < 0.01$) (Table 2).

Discussion

The kynurenic pathway, which produces kynurenic acid among other products, is a crucial link between the immune and the nervous system. This pathway plays a particularly complex and key role in immunoregulation during infections, pregnancy, autoimmune reactions, neoplasia and after organ transplantations. A more intensive catabolism of tryptophan on the kynurenic pathway appears under the influence of inflammation mediators, such as interferon γ (IFN-γ), and some of the pathway metabolites (kynurenines) produce a feedback immunosuppressive effect through the inhibition of the synthesis of cytokines. Increased metabolism of tryptophan in the kynurenic pathway may also inhibit the proliferation of activated immunocompetent cells, e.g. T cells, locally, in the site of inflammation, and systemically, in the whole body [2, 13, 14]. This effect is observed during gestation, when inhibition of the response of T cells is essential to carry a foetus to term, and is associated with the development of immunologic tolerance. Therefore, some authors worry that prolonged, chronic stimulation of the degradation of tryptophan on the kynurenic pathway may result in an undesirable depression of the activity of T cells and growing immunologic deficit [13]. Kynurenic acid is one of the kynurenines which produce an anti-inflammatory effect. The reference value of the KYNA concentration in a healthy man's plasma is measured in nanomoles, but rises to micromoles during inflammation. And it is this higher than the physiological level of KYNA that activates GPR35 receptors on cells of the immune system, producing an anti-inflammatory effect, mainly through the inhibition of secretion of pro-inflammatory cytokines (IL-1α, IFN-γ, TNF-α, HMGB1) by negative feedback [2, 5, 15-17].

The depressed proliferative response of splenocytes found in the fish which received the highest dose of KYNA in feed for 7 days is therefore attributable to the mentioned suppressive/anti-inflammatory effect of KYNA. However, this was a short-lived effect and, as the administration of the acid was continued, the proliferative activity of splenocytes in this group of fish increased up to the level recorded in the control group. The lack of any effect produced by the intermediate KYNA dose on the proliferative response

of splenocytes is not surprising. It may have been due to the inability to reach a sufficiently high level of the acid to activate GPR receptors on the surface of the spleen's lymphocytes when the rate of the acid in feed was so low. Similar results were reported by Kudo *et al.* [14] from their *in vivo* experiments with different kynurenines; unlike other metabolites of the kynurenic pathway, kynurenic acid did not have any influence on PHA-stimulated mononuclear leucocytes in human peripheral blood. However, it is difficult to explain why the proliferation of T cells was stimulated after 28 days of the administration of the lowest dose of KYNA to fish.

Another parameter determined in fish was the effect of kynurenic acid on the activity of phagocytic cells. This development is rather scantily described in the literature and results of different studies are unequivocal. It is known that KYNA inhibits secretion of typical pro-inflammatory cytokines of macrophages (TNF-α), and it also depresses the release of HNP1-3α-defensin, which is recognized as an important marker of the activation of granulocytes, associated with an efficient destruction of microorganisms by these cells [5, 17]. On the other hand, Barth *et al.* [18] demonstrated an unquestionably stimulating effect of kynurenic acid on adhesion of monocytes and human neutrophils to the vascular endothelium, which – as the cited authors claim – suggests that KYNA can act as a chemokine and represents an important agent in the early stage of activation of phagocytic cells. Thus, the effect of KYNA on the immune system might be more complex, including an early pro-inflammatory phase (positive effect on adhesion of phagocytes) and a later anti-inflammatory phase (inhibition of the synthesis of pro-inflammatory cytokines). The present report verifies the stimulating effect of the short-term administration of kynurenic acid on the activity of phagocytic cells in fish.

Recapitulating, kynurenic acid administered to rainbow trout orally affected the activity of immunocompetent cells isolated from the spleen of these fish. The most profound effect appeared after the shortest tested period of the administration of the highest KYNA dose. However, this was not an univocal effect – the depressed proliferative response of lymphocytes was observed simultaneously with a more intensive activity of phagocytic cells. Considering the dominant role of non-specific immunity agents, mainly phagocytic cells, in the response of fish to pathogens, it is possible to claim that kynurenic acid may find applications in the prevention of diseases in aquaculture, once its optimal dose and administration period are established. For this aim, however, it will be necessary to conduct wider research on different species of cultured fish.

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