DOI: 10.1590/1809-6891v24e-73661E

Ciência Animal Brasileira
Brazilian Animal Science

e-ISSN 1809-6891

Section: Veterinary medicine
Research article

Effects of supplementation with vitamins C and E on the acute inflammatory response in Piractus mesopotamicus

Efeitos da suplementação com vitaminas C e E na resposta inflamatória aguda em *Piaractus mesopotamicus*

Fabiana Rizzo Bozzo[1], Gustavo da Silva Claudiano[2], Jefferson Yunis-Aguinaga[3], Paulo Fernandes Marcusso[4], Jair Rodini Engrácia Filho[1], Julieta Rodini Engrácia de Moraes[1]

[1]Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Jaboticabal, São Paulo, Brazil
[2]Universidade Federal do Oeste do Pará (UFOPA), Santarém, Pará, Brazil
[3]Universidad Científica del Sur, Lima, Peru
[4]Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, São Paulo, Brazil
*Autor correspondente: julietaengracia@gmail.com

Abstract
Vitamins C and E are potent antioxidants that reduces the harmful effects of stress in several species including fish. In this study, it was evaluated the effect of vitamins C, E and their combination in the acute aerocystitis induced by inactivated *Aeromonas hydrophila* in pacu. 288 fish were distributed into 4 groups supplemented for 90 days: G1-control; G2-supplemented with 500 mg of Vitamin C; G3-supplemented with 500 mg of Vitamin E; G4-supplemented with 500 mg of Vitamin C + 500 mg of Vitamin E. The fish were divided in three groups, the first was not inoculated; second were inoculated in the swim bladder with 3 x 10^9 CFU of inactivated *A. hydrophila* and the last one with saline. The inflammatory exudate was collected from the swim bladder for assessment of cellular component and cytochemistry. The results showed higher accumulation of leukocytes in fish inoculated with bacteria. Cytochemistry was effective identifying thrombocytes, lymphocytes, macrophages and, granulocytes present in the exudate. It was also observed fish that received supplementation with vitamins presented higher accumulation of total cells in the exudate with a predominance of lymphocytes and thrombocytes. These results suggested that supplementation with vitamins improved the immunological responses.
Keywords: *Aeromonas hydrophila*; cytochemistry; pacu; swim bladder.

Resumo
As vitaminas C e E são potentes antioxidantes que reduzem os efeitos nocivos do estresse em várias espécies, incluindo peixes. Neste estudo, avaliou-se o efeito das vitaminas C, E e sua combinação na aerocistite aguda induzida por *Aeromonas hydrophila* inativada em pacu. 288 peixes foram distribuídos em 4 grupos suplementados por 90 dias: G1-controle; G2-suplementado com 500 mg de Vitamina C; G3-suplementado com 500 mg de Vitamina E; G4-suplementado com 500 mg de Vitamina C + 500 mg de Vitamina E. Os peixes foram divididos em três grupos, o primeiro não foi inoculado; o segundo foi inoculado na bexiga natatória com 3 x 10^9 UFC de *A. hydrophila* inativada e a última com soro fisiológico. O exsudato inflamatório foi coletado da bexiga natatória para avaliação do componente celular e citoquímica. Os resultados mostraram maior acúmulo de leucócitos nos peixes inoculados com a bactéria. A citoquímica foi eficaz na identificação de trombócitos, linfócitos, macrófagos e granulócitos presentes no exsudato. Também foi observado que os peixes que receberam suplementação com vitaminas apresentaram maior acúmulo de células totais no exsudato com predominância de linfócitos e trombócitos. Esses resultados sugeriram que a suplementação com vitaminas melhorou as respostas imunológicas.
Palavras-chave: *Aeromonas hydrophila*; citoquímica; pacu; bexiga natatória.

1.Introduction

Vitamins are important micronutrients which are required for the maintenance of normal body functions. It plays an important role in the growth, immune response, hematology, reproduction, and response to stressors [(1)]. A proper amount of these micronutrients is required for the normal catalytic processes within the enzymatic system which consists of a variety of enzyme activities linked with the metabolic, endocrine and immune systems [(2)]. The deficiency of these micronutrients causes many metabolic disorders and diseases/infections through their negative influence on the physiological system in fish and other animals [(3,4)].

Vitamins C and E are important for improving the growth and physiological health of fish. These vitamins must be supplemented regularly in fish diets, as most teleost fish are unable to synthesize them [(5)]. Vitamin C (ascorbic acid) is a potent antioxidant that reduces the harmful effects of stress and the activity of inflammatory cells [(6)]. Vitamin E is also an important antioxidant that protects cell membranes from lipid peroxidation and enhances disease resistance [(7,8)]. Diets supplemented with vitamins C and E increased phagocytic activity and the production of ROS (reactive oxygen species) in kidney leukocytes of *Spaurus aurata* [(9)] and reduces the mortality by *Yersinia rukeri* in *Oncorhynchus mykiss* [(10)]. However, the use of high doses presented no benefits against

Received: August 8, 2022. Accepted: October 17, 2022. Published: December 29, 2022.

Streptococcus iniae infection [11].

Aeromonas hydrophila is a facultatively anaerobic, Gram-negative bacterium that infects a wide range of invertebrate and vertebrate hosts. In fish, it causes motile aeromonad septicemia [12]. Is considered one of the most important bacterial diseases responsible for the loss of millions of dollars in the global freshwater aquaculture industry [13]. Several antibiotics supplementations used to prevent or control bacterial infections have been banned in several countries, due to their increasingly negative consequences. Whereas alternative compounds are being applied to elevate the quality and sustainability of fish production [14,15].

Swim bladder has been used as a model for the study of fish inflammation due it is a cavity with terminal circulation with no resident leukocytes and presents easy access for inoculation and collection of exudate [16,17]. Therefore, a great tool for evaluating the inflammation response, as well as its alterations and supplemented animals. Based on the above content this study, the effect of dietary supplementation with vitamins C, E and their combination on the acute aerocystitis induced by inactive *Aeromonas hydrophila* in *Piaractus mesopotamicus* was evaluated.

2.Material and methods

2.1 Experimental design and diets

This experiment was carried out with 288 pacus, *Piaractus mesopotamicus* (197.1 ± 21.5 g), in 48 tanks (250 L / n = 6) with water flow of 1 L min^{-1} and continuous aeration. Water quality remained at the comfort zone of this species (Dissolved oxygen= 5.3 ± 0.3 mg L^{-1}, Temperature = 29.4 ± 1.5°C, pH = 7.6 ± 0.1 and electric conductivity = 117.9 ± 5.1 µS cm^{-1}). Ethical protocol for this study was approved by Ethics committee (CEUA-UNESP) under protocol number 003868/10 in accordance with guidelines for care and use of laboratory animals of Brazilian laws.

Fish were distributed randomly in four groups: G1 unsupplemented, control (commercial feed / Vit C 66.5 mg kg^{-1} and Vit E 14.1 mg kg^{-1}); G2 supplemented with 500 mg of Vitamin C per kg of feed [18] G3 supplemented with 500 mg of Vitamin E per kg of feed [19] and G4 supplemented with 500 mg of Vitamin C + 500 mg of Vitamin E per kg of feed [19, 20].

It was used commercial feed for pacu (28% CP and 3900 kcal kg^{-1} DE) (Table 1). Ascorbyl polyphosphate-35% of activity was used as source of vitamin C and α-tocopheryl-50% activity as source of vitamin E. Fish were fed twice a day (3% of biomass) for 90 days [21]. The vitamins were incorporated during the second milling of the extrusion process. The ingredients were mechanically mixed in the treatments.

Table 1. Percentage composition of experimental diets.

Ingredients	%
soybean meal	43
Corn	22.6
Wheat bran	17
Rice bran	10
yeast	4
L-lysine	0.2
DL-methionine	0.4
dicalcium phosphate	1
limestone	1
vitamin and mineral premix (without vitamin C and E)	0.5

*Vitamin and mineral premix supplied by the company FRI-RIBE. Vitamin C and E were added in the treatment (500 mg of tocopheryl acetate and 500 mg of ascorbic acid / Kg of food)

At the end of the experiment, vitamins were quantified in the diets, for vitamin C (489 mg kg^{-1}) was used titration [22] and for vitamin E (484 mg kg^{-1}) was used HPLC [23]. Control group concentration of vitamin C was 66.5 mg kg^{-1} and vitamin E 14.1 mg kg^{-1} confirmation described above.

2.2 Induction and evaluation of acute aerocystitis by A. hydrophila

Swim bladder was chosen as a model to study inflammation due to it is a cavitary organ with terminal circulation easy for inoculation and collection of material to evaluate cellular and fluid components in the inflammatory focus. Other important feature is that has no resident leukocytes [16].

Fish were anesthetized in benzocaine solution (1: 20 000) in 98° ethanol (0.1 g mL^{-1}) [16]. After anesthesia, the fish the of each treatments (G1 – G4) were divided in three subgroup, the first was not inoculated (NV); the second was inoculated in the swim bladder, anteromedial region (1cm from operculum), on the lateral line, with a sterile needle and syringe, with $3x10^9$ UFC de heat-inactivated *Aeromonas hydrophila* in 1.0 mL of 0.65% saline solution (Bacteria) and the last subgroup injected with the same volume of 0.65% saline solution. This dose was previously determined by LD50 assay. After 4, 24 and 48 hours post inoculation (HPI), 6 fish per treatment and time were sacrificed under deep anesthesia. The swim bladder was washed with 0.5 mL of phosphate buffered saline with 0.01 mL of EDTA 5% and cell suspensions were centrifuged at 300 g for 10 minutes before staining. Total number cell count was determined with a Neubauer haemocytometer [16].

Exudate smears were air-dried, fixed and stained with Giemsa or periodic acid-Schiff (PAS). The percentages of lymphocytes, granulocytes, macrophages, and thrombocytes were determined according to Claudiano et al. [16] and cells were measured by image analyzer Video Plan (Kontron Elektronik Zeiss, Germany) [21].

2.3 Cytochemical characterization of the cellular component

Exudate smears were treated by PAS staining to identify the glycogen [24] and then, positive stained smears were treated by salivary amylase to confirm the presence of glycogen to Tavares-Dias et al. [25, 26]. The identification of peroxidase and nonspecific esterase was performed using o-toluidine-hydrogen peroxide Tavares-Dias et al. [25, 26].

2.4 Statistical analysis

The results were subjected to analysis of variance and comparison of means through Tukey test at a significance level of 5%.

3. Results

3.1 Evaluation of acute aerocystitis by Aeromonas hydrophila

Fish inoculated with inactivated *A. hydrophila* showed higher accumulation of total cells (P <0.05) than those injected with saline (Table 2). The groups inoculated with bacteria and supplemented with vitamin E and C + E showed increased accumulation of total cells (P <0.05) than unsupplemented and supplemented with vitamin C after 24 h. The accumulation of total cells was higher (P <0.05) 48 h after inoculation with *A. hydrophila* in fish supplemented with vitamin E and C + E, compared to unsupplemented or injected with saline with or without vitamins (Table 2). Non-inoculated fish showed no resident cell in the swim bladder.

Table 2. Means[1] (standard deviation) and analysis of variance [2] of the accumulation of inflammatory cells in the swim bladder of pacus supplemented with 500 mg/ kg diet of vitamin C, E, the association and inoculated with *A. hydrophila*

	Treatments	variable	Total cells				Thrombocytes				Lymphocytes				Granulocytes				Macrophages			
4h	G1	NV	0	±	0	Ca	0	±	0	Ba	0	±	0	Ca	0	±	0	Ba	0	±	0.03	Aa
		Saline	60.3	±	20.1	Ba	19.7	±	5.63	Aa	24.5	±	5.2	Bb	12.9	±	0.07	Aa	3.1	±	0.03	Aa
		Bacteria	373.5	±	60.5	Aa	60.3	±	3.01	Aa	290.5	±	9.3	Aa	18.7	±	0.03	Aa	4	±	0.07	Aa
	G2	NV	0	±	0	Ca	0	±	0	Ba	0	±	0	Ca	0	±	0	Ba	0	±	0	Aa
		Saline	58.3	±	5.9	Bb	10.3	±	1.83	Aba	50.7	±	1.83	Ba	9.4	±	1.83	Aa	5	±	1.83	Aa
		Bacteria	183.4	±	30.6	Aa	25.3	±	1.91	Aa	133.1	±	10	Aa	20.7	±	1.91	Aa	4.3	±	1.91	Aa
	G3	NV	0.9	±	2.56	Ca	0	±	0	Ba	0	±	0	Ca	0	±	0	Ba	0	±	0	Aa
		Saline	71.8	±	10.3	Bb	18.7	±	1.83	Aa	39.7	±	5.9	Ba	17.19	±	1.83	Aa	2.5	±	1.83	Aa
		Bacteria	219.1	±	50.8	Aa	22.9	±	1.91	Aa	173.4	±	20.7	Aa	18.9	±	1.91	Aa	3.9	±	1.91	Aa
	G4	NV	0	±	0	Ca	0	±	0	Ba	0	±	0	Ca	0	±	0	Ba	0	±	0	Aa
		Saline	40.1	±	3.9	Bb	10.5	±	2.68	Ba	45.7	±	20.1	Ba	5.7	±	0.3	Aa	4.8	±	0.11	Aa
		Bacteria	488.8	±	70.1	Aa	80.7	±	10.2	Aa	380.3	±	95.5	Aa	24.7	±	0.6	Aa	3.1	±	0.04	Aa
24h	G1	NV	0	±	0	Ca	0	±	0	Ca	0	±	0	Ca	0	±	0	Ba	0	±	0	Ba
		Saline	80.9	±	1.91	Bb	21.44	±	5.63	Ba	50.7	±	4.7	Ba	10.9	±	0.7	Aa	1.9	±	0.03	Ba
		Bacteria	814.7	±	10.7	Ab	380.9	±	20.3	Aa	380.7	±	20.8	Ab	14.7	±	0.3	Aa	38.4	±	17.3	Aa
	G2	NV	0	±	0	Ca	0	±	0	Ca	0	±	0	Ca	0	±	0	Ba	0	±	0	Ba
		Saline	90.3	±	1.91	Bb	27.3	±	1.83	Ba	20.9	±	1.83	Bb	19.7	±	1.83	Aa	3.4	±	1.83	Ba
		Bacteria	529.1	±	15.3	Ab	280.3	±	10.8	Aa	205.8	±	20.8	Ab	12.3	±	10.9	Aa	30.7	±	10.9	Aa
	G3	NV	0	±	0	Ca	0	±	0	Ca	0	±	0	Ca	0	±	0	Ba	0	±	2.56	Ba
		Saline	83.8	±	1.9	Bb	30.9	±	1.83	Ba	20.7	±	3	Ba	16.9	±	5.7	Aa	4.9	±	1.83	Ba
		Bacteria	1118.7	±	30.9	Aa	578.9	±	1.91	Aa	450.9	±	1.91	Aa	28.5	±	30.7	Aa	60.4	±	20.3	Aa
	G4	NV	0	±	0	Ca	0	±	0	Ca	0	±	0	Ca	0	±	0	Ba	0	±	0	Ba
		Saline	79.3	±	5.8	Bb	40.7	±	2.68	Ba	690.8	±	90.7	Bb	15.7	±	2.9	Aa	5.4	±	0.11	Ba
		Bacteria	1075.9	±	25.9	Aa	410.7	±	10.9	Aa	600.7	±	20.9	Aa	18.3	±	20.7	Aa	45.7	±	4.3	Aa
48h	G1	NV	0	±	0	Ca	0	±	0	Ca	0	±	0	Ca	0	±	0	Ba	0	±	0	Ba
		Saline	40.7	±	1.91	Bb	21.44	±	5.63	Ba	60.7	±	20.5	Ba	10.9	±	5.7	Aa	2.9	±	0.03	Ba
		Bacteria	1111.6	±	350.4	Ab	800.7	±	30.9	Aa	200.8	±	90.5	Aa	19.3	±	50.4	Aa	90.8	±	5.9	Aa
	G2	NV	0.19	±	0.09	Ca	0	±	0	Ca	0	±	0	Ca	0	±	0	Ba	0	±	0	Ca
		Saline	30.8	±	10.7	Bb	3.4	±	1.83	Bb	70.8	±	10.9	Ba	30.1	±	1.83	Aa	6.1	±	1.83	Ba
		Bacteria	2624.2	±	400.9	Aa	2000.4	±	20.1	Aa	500.7	±	30.5	Aa	44.7	±	60.7	Aa	78.4	±	4.3	Aa
	G3	NV	0	±	0	Ca	0	±	0	Ca	0	±	0	Ca	0	±	0	Ba	0	±	2.56	Ba
		Saline	29.7	±	20.1	Ba	9.7	±	80.2	Bb	80.7	±	60.2	Ba	7.19	±	1.83	Aa	4.9	±	1.83	Ba
		Bacteria	2120.6	±	50.9	Aa	1450.1	±	20.3	Aa	590.3	±	100.2	Aa	42.1	±	160.7	Aa	38.1	±	10.9	Aa
	G4	NV	0	±	0	Ca	0	±	0	Ca	0	±	0	Ca	0	±	0	Ba	0	±	0	Ba
		Saline	30.9	±	14.7	Ba	15.7	±	2.68	Ba	75.4	±	30.5	Ba	24.7	±	3.9	Aa	2.4	±	0.11	Ba
		Bacteria	1834.6	±	150.7	Aa	1220.1	±	500.3	Aa	552.9	±	20.9	Aa	21.9	±	105.4	Aa	39.7	±	4.8	Aa

Means (n=6) with the same letter are not significantly different by Tukey test (P>0.05) [2] Capital letters compare the types of inoculum and lowercase letters compare different groups. G1 non-supplemented, control; G2 supplemented with 500 mg of vitamin C; G3 supplemented with 500 mg of vitamin E and G4 supplemented with 500 mg of vitamin C + 500 mg of vitamin E /kg of feed. NV - not inoculated; Saline - 0.65%; Bacteria - inoculated with 3x 10^9 CFU of *A. hydrophila* h= hours after inoculation.

After four hours, it was observed no difference in the number of thrombocytes, lymphocytes, granulocytes and macrophages between supplemented and unsupplemented groups. In the group supplemented with vitamin C + E the accumulation of thrombocytes was higher (P <0.05) in fish inoculated with the bacteria in relation to fish injected with saline. After 24 h, fish inoculated with *A. hydrophila* showed greater accumulation of thrombocytes than saline injected fish (p <0.05) and there was no difference between supplemented groups (p> 0.05). The accumulation of thrombocytes at 48 h in fish inoculated with bacteria and supplemented with vitamins C, E and C + E was higher (P <0.05) than in non-supplemented fish (Table 2).

Fish inoculated with bacteria showed higher accumulation of lymphocytes (p<0.05) than those injected with saline in all times. After 4h after injection of saline in supplemented fish the accumulation of lymphocytes was higher than in non-supplemented fish. After 24 h the accumulation of lymphocytes was higher (p>0.05) in supplemented with vitamin E and C + E (G3 and G4) than those supplemented with vitamin C and not supplemented (G1 and G2). After 48h, the accumulation of lymphocytes was higher (P <0.05) in the group inoculated with bacteria and supplemented with vitamins comparing with non-supplemented group under the same inoculum (Table 2).

After 24 and 48 h, fish inoculated with the bacteria showed higher accumulation of macrophages than those injected with saline (p<0.05). There was no difference between supplemented groups in relation to non-supplemented (p> 0.05) (Table 2). There was no difference (p> 0.05) in granulocytes in all groups and times different groups (Table 2).

3.2 Cytochemical analysis of the cellular component

There was observed macrophages, thrombocytes, granulocytes, and lymphocytes in the inflammatory exudate (Figure 1). Lymphocytes and thrombocytes stained strongly with PAS. Monocytes were also stained but faintly. Lymphocytes, macrophages and granulocytes were positive to o-toluidine-hydrogen peroxide. Nonspecific esterase reaction was observed as dark granules within the cytoplasm of macrophages and thrombocytes (Figure 1).

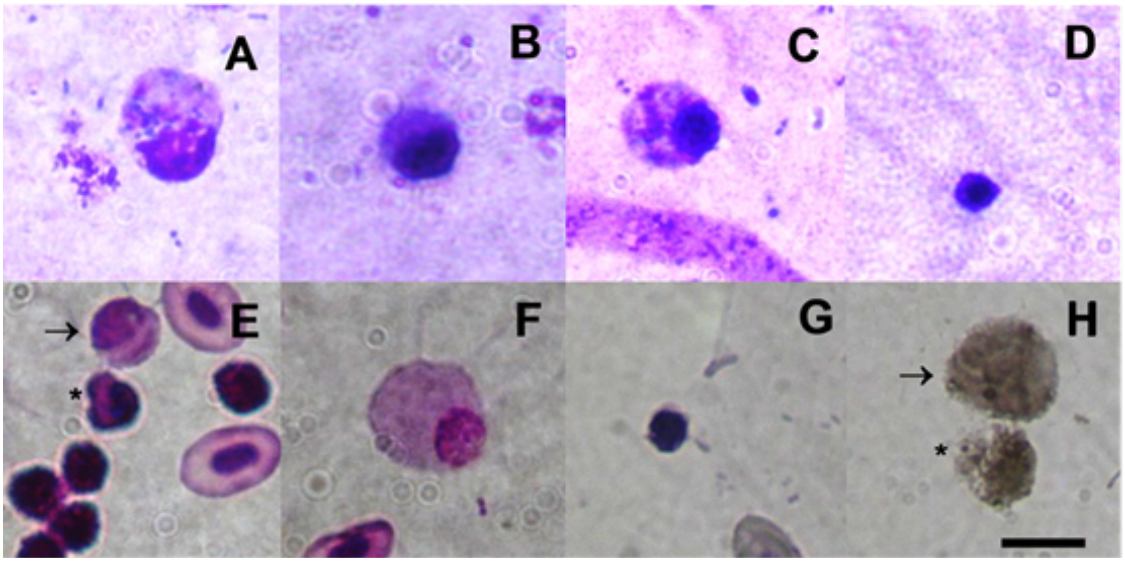

Figure 1. Photomicrograph of leukocytes from the exudate: (A) macrophages, (B) thrombocytes, (C) granulocyte, (D) lymphocytes; stained with May-Grunwald-Giemsa-Wrigth. (E) arrow: macrophages, asterisk: lymphocytes; (F) granulocyte; stained with ortho-toluidine; (G) lymphocytes; stained with PAS; (H) arrow: macrophages, asterisk: thrombocytes; staining with nonspecific esterase. Bar = 25 µm.

4. Discussion

The solid results obtained in this work support the use of swim bladder as a model for fish inflammation studies. Other authors have validated the use of this model [16,17,21,27,28]. However, to our knowledge, this is the first time it is characterized the cellular component of the exudate by cytochemistry after vitamin C and E supplementation on *Piaractus mesopotamicus.* Fish inoculated with inactivated *A. hydrophila* showed higher accumulation of total cells than injected with saline and not treated fish, in all times, proving the efficiency of the experimental model.

The results of this study also showed that the morphological analysis and cytochemistry were effective to identify the cell types of the exudate. In fish supplemented with vitamins C, E and its association did provoke accumulation of total cells in the inflammatory site, mainly thrombocytes and lymphocytes, which indicates a more efficient response.

Li et al. [29] showed that, unlike mammals, B-lymphocytes of primitive animals have a potent *in vitro* and *in vivo* phagocytic activity. This suggests that these cells may have originated from one cell type with phagocytic activity [30]. In this study, lymphocytes showed a positive cytochemical reaction for glycogen granules which were stained by PAS and peroxidase. These findings support that lymphocytes have evolved from a myeloid ancestor and possess enzymes to phagocytic activity. As expected, macrophages and granulocytes were also peroxidase-positive.

Thrombocytes were nonspecific esterase and PAS-positive. Similar results were observed in *Oncorhynchus mykiss* [31]. This can be attributed to its role as an active phagocytic cell [26]. The qualitative analysis of the inflammatory cell component in not supplemented fish corroborates the results of Bozzo et al. [28] where thrombocytes and lymphocytes were predominant. This support that both have an important role in defense against pathogens, as previously demonstrated by Tavares-Dias et al [26].

Supplementation with vitamins C, E and C + E enhance the response in at least one of the assessments. Dietary supplementation with vitamin C in bacteria-inoculated fish potentiated the inflammatory response corroborating previous findings in chronic inflammation [18, 20] in the same species and improved immune response and growth in fish [8].

Fish supplemented with vitamin E and inoculated with the bacteria presented higher total number of cells in the swim bladder compared to non-supplemented group. Vitamin E is a potent antioxidant that inhibits the action of free radicals and enhances disease resistance [7]. Its deficiency enhances the harmful effects of stress in *P. mesopotamicus* kept at high stocking density [32].

Pacu fed diet deficient in vitamin E show a negative correlation between the number of monocytes and plasma cortisol, indicating the susceptibility to the suppressive effect of glucocorticoids [19]. High levels of circulating cortisol decrease leukocyte adhesion to vascular endothelium, diapedesis, and chemotaxis of monocytes by inhibition of eicosanoid synthesis [16].

We observed that fish supplemented with the combination of vitamin C + E increase the number of total cells compared to non-supplemented and control groups. The effect was significant after 4 HPI in fish inoculated with *A. hydrophila* compared to those who received vitamin C or E. The same occurred with thrombocytes after 48 HPI.

Martins et al. [33] examined the effects of dietary supplementation with 500 mg of vitamin C, associated with the same concentration of vitamin E in acute inflammation induced by carrageenan in pacus. Supplemented fish had higher accumulation of total cells in the inflamed site, particularly thrombocytes compared to non-supplemented group, corroborating the current results.

5. Conclusion

Supplementation with 500mg/kg of vitamin C and E empacus generated greater accumulation of cells at the immunological site of inflammation in A. Hydrophila and, therefore, helped the vitamin system of these species.

Conflict of interests

The authors declare no conflict of interest.

Author contributions

Conceptualization: F. R. Bozzo, G. S. Claudiano and J. R. E. de Moraes. *Data curation*: F. R. Bozzo and G. S. Claudiano. *Formal Analysis*: F. R. Bozzo, G. S. Claudiano, J. Yunis-Aguinaga, P. F. Marcusso and J. R. E. Filho. *Investigation*: F. R. Bozzo, G. S. Claudiano, J. Yunis-Aguinaga, P. F. Marcusso and J. R. E. Filho. *Methodology*: F. R. Bozzo, G. S. Claudiano, J. Yunis-Aguinaga, P. F. Marcusso and J. R. E. Filho. *Funding acquisition*: J. R. E. de Moraes. *Project administration*: J. R. E. de Moraes. *Supervision*: J. R. E. de Moraes. *Writing (original draft):* F. R. Bozzo, G. S. Claudiano and P. F. Marcusso. *Writing (review & editing):* F. R. Bozzo, G. S. Claudiano, J. Yunis-Aguinaga, P. F. Marcusso and J. R. E. de Moraes

Acknowledgments

The authors thank Research Support State of São Paulo Research Foundation (04/13081-0 and 05/55788-5) and CNPq (National Counsel of Technological and Scientific Development) for financial support to J.R.E. Moraes and F.R. Moraes. *In Memoriam*: A Great Friend and Mentor, Professor Flávio Ruas de Moraes

References

1. R. E Ibrahim, S.A. Ahmed, S.A Amer, N.A Al-Gabri, A.I Ahmed, A.A. Abdel-Warith, M.I. Younis, A.E. Metwally Influence of vitamin C feed supplementation on the growth, antioxidant activity, immune status, tissue histomorphology, and disease resistance in Nile tilapia, *Oreochromis niloticus*. Aquaculture Reports, 18 (2020), p. 100545

2. Keen CL, Uriu-Adams JY, Emsimsa JL, Gershwin ME. Trace elements/minerals and immunity. In: Gershwin, M.E., Nestel, P., Keen, C.L., Totowa, N.J. (Eds.), Handbook of Nutrition and Immunity. Humana Press, 2004; 117–140.

3. Ekiz C, Agaoglu L, Karakas Z, Gurel N, Yalcin I. The effect of iron deficiency anemia on the function of the immune system.

Hematol. J. 2005; 5, 579–583.

4. Koshio S, Angeles M. Beneficial roles of feed additives as immunostimulants in aquaculture: a review Rev. Aquac. 2017; 1–25. https://doi.org/10.1111/raq.12209

5. Khan, K.U., Zuberi, A., Nazir, S., Fernandes, J.B.K., Jamil, Z., Sarwar, H. Effects of dietary selenium nanoparticles on physiological and biochemical aspects of juvenile Tor putitora, Turk. J. Zool. 2016. 40, 704–712.

6. Liang XP, Li Y, Hou YM, Qiu H, Zhou QC. Effect of dietary vitamin C on the growth performance, antioxidant ability and innate immunity of juvenile yellow catfish (*Pelteobagrus fulvidraco* Richardson). Aquac. Res. 2017; (48): 149-160.

7. Etsuo N. Lipid oxidation that is, and is not, inhibited by vitamin E: Consideration about physiological functions of vitamin E. Free Radical Biology and Medicine. 2021; 176(20): 1-15.

8. Pan JH, Feng L, Jiang WD, Wu P, Kuang SY, Tang L, Zhang YA, Zhou XQ, Liu Y. Vitamin E deficiency depressed fish growth, disease resistance, and the immunity and structural integrity of immune organs in grass carp (*Ctenopharyngodon idella*): Referring to NF-κB, TOR and Nrf2 signaling. Fish and Shellfish Immunology. 2017; 60:219-236. Disponível em: 10.1016/j.fsi.2016.11.044

9. Ortuño J, Cuesta A, Esteban MA, Meseguer J. Effect of oral administration of high vitamin C and E dosages on the gilthead seabream (*Spaurus aurata L.*) innate immune system. Veterinary Immunology and Immunopathology. 2001. 200; 79(3-4): 167-180. Disponível em: 10.1016/s0165-2427(01)00264-1

10. Wahli T, Verlhac V, Gabaudan J, Schuep W, Meier W. Influence of combined vitamins C and E on non-specific immunity and disease resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases. 1998; 21(2): 127–137. Disponível em: 10.1046/j.1365-2761.1998.00088.x

11. Sealey WM, Gatlin DM. Dietary vitamin C and vitamin E interact to influence growth and tissue composition of juvenile hybrid striped bass (*Morone chrysops* (female) x *M. saxatilis* (male) but have limited effects on immune responses. The Journal of Nutrition 2002; 132(4): 748–755. DOI: 10.1093/jn/132.4.748

12. Le TS, Nguyen TH, Vo HP, Doan VC, Nguyen HL, Tran MT, Tran TT, Southgate PC, Kurtboke DI. Protective fffects of bacteriophages against Aeromonas hydrophila species Causing Motile Aeromonas Septicemia (MAS) in striped catfish. Antibiotics (Basel). 2018; 7(1). https://doi.org/10.3390/antibiotics7010016

13. Peterman MA, Posadas BC. Direct economic impact of fish diseases on the East Mississippi catfish industry. North American Journal of Aquaculture. 2019; 81(3): 222–229. https://doi.org/10.1002/naaq.10090

14. Kuebutornye FKA, Abarike ED. The contribution of medicinal plants to tilapia aquaculture: a review Aquac. Int. 2020; (28): 965-983. https://doi.org/10.1007/s10499-020-00506-3

15. Thépot V, Campbell AH, Rimmer MA, Paul NA. Meta-analysis of the use of seaweeds and their extracts as immunostimulants for fish: a systematic review Rev. Aquac. 2021; (13): 907-933,

16. Claudiano GS, Yunis-aguinaga J, Marinho-neto, FA, Miranda, R, Martins, IM, Otani FS, Mundim AV, Marzocchi-machado CM, Moraes JRE, de Moraes, FR. Hematological and immune changes in Piaractus mesopotamicus in the sepsis induced by Aeromonas hydrophila. Fish & Shellfish Immunology. 2019; 88: 259-265.

17. Yunis-Aguinaga J, Claudiano GS, Marcusso PF, Manrique WG, De Moraes JRE, De Moraes FR, Fernandes JB. *Uncaria tomentosa* increases growth and immune activity in *Oreochromis niloticus* challenged with *Streptococcus agalactiae*. Fish and Shellfish Immunology. 2015; 47(1), 630-638. Disponível em: https://doi.org/10.1016/j.fsi.2015.09.051

18. Moraes JR, Freitas JB, Bozzo FR, Moraes FR, Martins ML. A suplementação alimentar com vitamina c acelera a evolução do processo cicatricial em P*iaractus mesopotamicus* (Holmberg, 1887). Boletim do Instituto de Pesc. 2003;29:57 – 67.

19. Belo MAA, Moraes FR, Yoshida L, Prado EJR, Moraes JRE, Soares VE, Silva MG. Deleterious effects of low level of vitamin E and high stocking density on the hematology response of pacus, during chronic inflammatory reaction. Aquaculture. 2014; 423:124–128. Disponível em: https://doi.org/10.1016/j.aquaculture.2013.12.013

20. Belo MAA, Moraes JRE, Soares VE, Martins ML, Brum CD, Moraes FR. Vitamin C and endogenous cortisol in foreign-body inflammatory response in pacus. Pesquisa Agropecuária Brasileira. 2012; 47(7): 1015-1021. Disponível em: https://doi.org/10.1590/S0100-204X2012000700019

21. Castro MP, Claudiano GS, Bortoluzzi NL, Garrido E, Fujimoto RY, Belo MAA, Shimada MT, Moraes JRE, Moraes FR. Chromium carbochelate dietary supplementation favored the glucocorticoid response during acute inflammation of *Piaractus mesopotamicus*. Aquaculture. 2014a; 432:114–118a. Disponível em: https://doi.org/10.1016/j.aquaculture.2014.04.036

22. Tavares-Dias M. Cytochemical method for staining fish basophils. Journal of Fish Biology. 2006b; 69(1): 312-317b. Disponível em: https://doi.org/10.1111/j.1095-8649.2006.01106.x

23. Pregnolatto W, Pregnolatto NP. Normas analíticas do Instituto Adolfo Lutz: métodos químicos e físicos para análise de alimentos. 3.ed. São Paulo: Instituto Adolfo Lutz; 1985. 533p.

24. Mcmanus JFA. Histological demonstration of mucin after periodic acid. Nature, 1946; 158 (4006):202.

25. Tavares-Dias MA. Morphological and cytochemical study of erythrocytes, thrombocytes and leukocytes in four freshwater teleosts. Journal of Fish Biology. 2006a; 68(6):1822–1833a. Disponível em: https://doi.org/10.1111/j.1095-8649.2006.01089.x

26. Tavares-Dias M, Ono EA, Pilarski F, Moraes FR. Can thrombocytes participate in the removal of cellular debris in the blood circulation of teleost fish? A cytochemical study and ultrastructural analysis. Journal of Applied Ichthyology. 2007; 23(6): 709-712. Disponível em: https://doi.org/10.1111/j.1439-0426.2007.00850.x

27. Castro MP, Claudiano GS, Petrillo TR, Shimada MT, Belo MAA, Marzocchi-Machado CM, Moraes JRE, Manrique WG, Moraes FR. Acute aerocystitis in Nile tilapia bred in net cages and supplemented with chromium carbochelate and *Saccharomyces cerevisiae*. Fish Shellfish Immunol. 2014b; 36(1): 284 – 290b.

28. Bozzo FR, Moraes JRE, Moraes FR, Pereira GT, Tavares-Dias M, Onaka EM. Kinetics of cellular component in inflammatory response induced by different stimuli in the swim bladder of *Piaractus mesopotamicus*, Holmberg, 1887 (Characidae). Journal of the World Aquaculture Society. 2007; 38(2): 302-308. Disponível em: https://doi.org/10.1111/j.1749-7345.2007.00100.x

29. Li J., Barreda DR, Zhang YA, Boshra H, Gelman AE, Patra S, Tort L, Sunyer JO. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nature Immunology. 2006; 7(10): 1116-1124. Disponível em: https://doi.org/10.1038/ni1389

30. Randelli E, Buonocore F, Scapigliati G. Cell markers and determinants in fish immunology. Fish and Shellfish Immunology. 2008; 25(4): 326–340. Disponível em: https://doi.org/10.1016/j.fsi.2008.03.019

31. Shigdar S, Harford A, Ward AC. Cytochemical characterization of the leucocytes and thrombocytes from Murray cod (*Maccullochella peelii peelii*, Mitchell). Fish and Shellfish Immunology. 2006; 26(5): 731-736. Disponível em: https://doi.org/10.1016/j.fsi.2009.03.010

32. Belo MAA, Schalch SHC, Moraes FR, Soares VE, Otoboni AMMB, Moraes JER. Effect of dietary supplementation with vitamin E and stocking density on macrophage recruitment and giant cell formation in the Teleost Fish, *Piaractus mesopotamicus.* J Journal of Comparative Pathology. 2005; 54(2-3):133-146. Disponível em: https://doi.org/10.1016/j.jcpa.2005.04.004

33. Martins ML, Moraes FR, Fujimoto RY, Onaka EM, Bozzo FR, Moraes JRE. Carragenin induced inflammation in *Piaractus mesopotamicus* (Osteichthyes: Characidae) cultured in Brazil. Boletim do Instituto de Pesca. 2006; 32(1): 31-39.