Histopathological changes in surubim *Pseudoplatystoma coruscans* subjected to high levels of vitamin C

[*Alterações histopatológicas do surubim Pseudoplatystoma coruscans submetido a altos níveis de vitamina C*]

"Artigo Científico/Scientific Article"

Rodrigo Yudi **Fujimoto**[1], Rudã Fernandes Brandão **Santos**[2], Claucia Aparecida **Honorato**[3*], Claudinei **Cruz**[4], Dalton José **Carneiro**[5]

[1]EMBRAPA-Tabuleiros Costeiros, Aracaju-SE, Brasil.
[2]Laboratório de Enzimologia, Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife-PE, Brasil.
[3]Faculdade de Ciências Agrarias, Universidade Federal da Grande Dourados (UFGD), Dourados-MS, Brasil.
[4]Curso de Agronomia, Centro Universitário da Fundação Educacional de Barretos, Barretos-SP, Brasil.
[5]Faculdade de Ciências Agrárias e Veterinárias, Campus de Jaboticabal, Departamento de Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Jaboticabal-SP, Brasil.
[*]Autor para correspondência/Corresponding author: E-mail: clauciahonorato@ufgd.edu.br

Abstract

This study evaluated the supplementation of vitamin C in diets for *Pseudoplatystoma coruscans* (surubim) juveniles. The effects of high concentrations of vitamin C were evaluated through the histological skin alterations regarding the structure of epidermis and collagen deposition. Ascorbic polyphosphate (AP) was used as a source of vitamin C in the diet of the surubim juveniles at concentrations of 500, 1000, 1500, 2000 and 2500 mg of AP/kg diet added to a basic diet (control; 0 mg AP/kg diet). After 90 days, six animals per treatment were euthanized and fragments of skin were sampled. The histological cuts were stained with hematoxilin - eosin (HE), Masson tricromic (TM), and Periodic acid Schiff (PAS). The epidermis showed disarrangement of the club cells in fish from control group and in fish fed with the 2000 and 2500 mg of AP/kg of diet. The treatment with 2500 mg of AP/kg of diet also caused the lowest epidermis height. However, no morphological skin alterations were observed on treatments 500, 1000, and 1500 mg of AP/kg of diet. Thus, decreases in epidermis height and changes in club cells arrangement can be recognized as new clinical signs of hypervitaminosis C in that fish, hindering skin development.

Keywords: feed; spotted; nutrition; tegument; vitamin C toxicity.

Resumo

A suplementação dietética de vitamina C é necessária para os peixes que não a sintetizam. A vitamina C é importante para sintetizar o colágeno e a formação de outros tecidos do corpo. Estudos de morfologia de pele são importantes para avaliar o estresse ambiental, má nutrição e intoxicação. O objetivo deste estudo foi avaliar o ascorbil polifosfato (AP) como fonte de vitamina C na dieta para juvenis de surubim (*Pseudoplatystoma coruscans*) por meio da morfologia da pele. Cinco dietas foram preparadas contendo 500, 1000, 1500, 2000 e 2500 mg de AP/kg de dieta e uma dieta sem AP. A morfologia da pele de peixes alimentados com 500 mg de dieta AP/kg ou mais, revelou uma intensa síntese de colágeno na derme. Nos tratamentos sem vitamina e em níveis mais elevados de inclusão 2000 e 2500 mg AP/kg de dieta ocorreu um desalinhamento celular em células basais da epiderme. O nível de 500mg AP/kg de dieta foi o nível ideal para prevenir os sinais clínicos de escorbuto e danos para o desenvolvimento dos animais. A hipervitaminose foi observada em nível de 2500mg AP.

Palavras-chave: alimentação; surubim; nutrição; tegumento; toxicidade de vitamina C.

Introduction

Vitamin C is essential in fish feeding because many species do not produce ascorbic acid due to a lack of the enzyme gulonolactone oxidase (O'keefe, 2001; Pezzato et al., 2004). This vitamin is an important factor in many reactions to maintain normal growth such as the synthesis of collagen

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from prolin hydroxylation; tryptophan hydroxylation to 5-hydroxytryptophan; and the conversion of 3,4-dihidroxyphenylpiruvate to norepinephrine (Dabrowski, 2001; Barros et al., 2002). Moreover, vitamin C is involved in immunological responses protecting the organism against injuries and infections.

Large doses of vitamin C have been used in prophylaxis and treatment of several diseases (Combs Jr., 2012). This practice was introduced in the 60s by Irwin Stone and Linus Pauling, suggesting that high doses of Vitamin C could control the occurrence of the common cold; several studies have since been conducted to test this hypothesis.

Several studies have been performed concerning the effects of high concentrations of vitamin C in different groups of animals; these studies predominantly reported its beneficial effects (Eicher et al., 2006). Nevertheless, there is not a unanimous opinion about the effects of high concentrations of vitamin C (Combs Jr., 2012). Overall, the results that obtained in fish show a small improvement in the expected index for growth and animal well-being (Chagas and Val, 2003). However, the different results attributed to the increase in vitamin C in the diets is supported by subjective data resulting from the meta-analysis conducted in the biological assays (Douglas et al., 2007; Combs Jr., 2012).

There is strong evidence that the intake of high concentrations of vitamin C by fish, and for long periods, may be toxic (Ortuno et al., 1999; Misra et al., 2007). These results are relevant because they are based on a large number of studies with the aim to demonstrate the positive effect of high concentrations of vitamin C in fish (Fujimoto and Carneiro, 2001; Tewary and Patra, 2008; Darias et al., 2011).

This study evaluated the supplementation of ascorbil polyphosphate as the source of vitamin C in diets formulated for *Pseudoplatystoma coruscans* spotted juveniles. This species had already been shown to be sensitive to toxicity from megadoses of vitamin C by Fujimoto and Carneiro (2001). In this study, the effects of high concentrations of vitamin C were evaluated through the analyses of histological skin alterations regarding the structure of epidermis and collagen deposition.

Material and Methods

Fingerlings of surubim *Pseudoplatistoma corruscans* were placed in 500L tanks for two weeks to recover and adapt to the new conditions. After such period, fish were slightly sedated with benzocaine (0.025 gL^{-1}) exposure for 1min; classified by weight range into 1g (small), 3g (medium) and 6g (large); and distributed in eighteen 100L-tanks. Three rows of six tanks received 42 fish of each weight class per row, seven fish per tank, and 21 fish per column or experimental condition, (N = 126). Each fish was considered an experimental unit (n = 21) and distributed into three weight classes to prevent cannibalism. The water was renewed 20 times a day, temperature was monitored daily and kept at 25ºC and oxygen level was weekly fixed at 5.1mgL^{-1}. The experimental conditions corresponded to six nutritional situations in which only the level of Vitamin C was changed: 0, 500, 1000, 1500, 2000, and 2500 mg of AP (ascorbyl-polyphosphate) per Kg diet (Table 1). Diet moisture level was adjusted to 40% in order to facilitate their acceptance and ingestion. After pelletized, the diets were packed and stored at -18ºC. The fingerlings were fed diets twice a day (8 PM and 7 AM) to reach satiety. Regarding the feeding, the water entering and aeration were discontinued, and the tanks briefly closed. Diets were offered in small amounts to prevent waste.

At the end of the experimental period, two fish from each tank (six animals per treatment) were sampled, euthanized with benzocaine (0.065 g L^{-1}), skin fragments were collected, fixed in Bouin solution for 24 hours, washed, dehydrated, and held in 70% ethanol. Fixed tissues were embedded in histosec (Merck) and cut into 5 µm slices. Tissue slices were mounted over glass slides, rehydrated, stained with hematoxylin-eosin (HE) or Masson's trichromic stain (MTS) and submitted to Periodic acid-Schiff (PAS) reaction. These procedures were done according to Genten et al. (2009). Five tissue areas from five fish from each treatment were evaluated under photomicroscope Olympus BX-41. The images were analyzed by the software Image pró-plus (Media - Cybernetics/USA) to evaluate epidermis and dermis. At first, normality of data were appraised and those not normal were transformed in a log set. Then, they were analyzed by ANOVA and Tukey tests ($p < 0.05$) to compare the means.

Table 1. Formulation and composition of the experimental diet (Basic diet).

Ingredients	% Diet
Fish meal	52
Soybean meal	21.5
Bran wheat	8
Corn	13.5
Soybean oil	4
Vitamin supplement *	0.5
Mineral supplement *	0.5
Calculated composition * *	
Crude protein (%)	40
Ether extract (%)	8.11
Crude fiber (%)	2.3
Crude energy (kcal/kg of diet)	4165
Relationship Ca: P	2:1

*Composition of the mineral and vitamin supplement: Iron 15000mg, Copper 5000 mg, Iodine 500 mg, Manganese 17000 mg, Zinc 12000 mg, Selenium 70 mg, vehicle 1000g, Vitamin TO 12000 UI, Vitamin D3 1500 UI, Vitamin and 50 mg, Vitamin K 4 mg, Vitamin B12 7 mg, Vitamin B2 7 mg, Pantothenic acid 60 mg, Nicotinic acid 120 mg, chloride of hill 600 mg, methionine 700 mg, antioxidant 500 mg, vehicle 1000g. **Calculated Composition with base in the analyses of food ingredients according to A.O.A.C. (1975).

The fish had difficulty to accept the dry diet, and so 40% of water was added and processed in a meat grinder. After pelletized, the diets were package and stored in freezer -18ºC, for conservation and to minimize losses.

The feeding was offered twice a day (at 8:00 and 19:00h). The diets were offer in small amounts to avoid losses but *ad libitum*, until the fish no more seek the food.

At the end of the experiment, 36 fish (six animals for each treatment) were euthanized with benzocaine (0.065.g.L^{-1}). Fragments of skin were collected, fixed in Bouin´s liquid for 24 hours washed and conserved in alcohol 705. The embed of material was made in histosec (Merck), and then histological cuts of 5 µm were realized. The histological cuts were submitted the stain with hematoxilin - eosin (HE), Masson tricromic (TM) and reacted in PAS. The coloration with TM was accomplished for detection of collagen. These procedures are in agreement with Genten et al. (2009).

In the end of the experiment five tissue areas from five fish each treatment were taken by photomicroscope Olympus BX-41. The images were analyzed by software Image pró-plus (Media – Cybernetics/USA) measuring the epidermis and dermis.

The data were submitted at ANOVA and Tukey test (at 5% of probability) for means differences.

Results

The following water parameters monitored during the experiment showed average dissolved oxygen of 5.5 ± 1.35 mg of O_2/L and an average temperature of 29 ± 1.5 ºC.

No morphological alterations were observed on the skin of surubim fed with 500, 1000, and 1500 mg of AP/kg of diet (Figure 1B, 1C, and 1D). The skin is constituted of the epidermis, dermis, and hypodermis. The epidermis is formed by the stratified epithelium divided into two layers: the external, constituted of non-keratinized cells with a central nucleus, uncondensed chromatin, and light cytoplasm, and the internal, constituted of basal cells with columnar shape (Malpighian cells). Mucus-secreting cells (PAS – positive) and club cells that produce a protein (glycoprotein) were also observed in the internal layer. This cell presents granules and shows a central nucleus and developed cytoplasm. The dermis is made up of the *stratum compactum* formed by collagen fibers.

The epidermis showed disarrangement in club cells in the fish fed without vitamin C (control) and fed with the 2000 and 2500 mg of AP/kg of diet (Figure 1A, 1E, and 1F); however, this alteration was not directly related to higher or lower epidermis height (Table 2). The treatment with 2500 mg of AP/kg of diet caused the lowest epidermis height.

Table 2. F value, coefficient of variation and mean epidermis height and collagen layer height in juveniles of surubim (*Pseudoplatystoma coruscans*) supplemented with vitamin C.

Statistics	Height of epidermis layer	Height of collagen layer
F to vitamin C	52.58 **	431.79 **
Coefficient of variation	3.54	2.72
0 mg/kg de vitamin C	4.275 ab	3.895 d
500 mg/kg de vitamin C	4.226 b	5.101 b
1000 mg/kg de vitamin C	4.015 c	5.315 a
1500 mg/kg de vitamin C	3.961 cd	4.754 c
2000 mg/kg de vitamin C	4.380 a	5.143 b
2500 mg/kg de vitamin C	3.888 d	4.790 c

An intense synthesis of collagen, mainly in the subepithelial layer, was observed in the dermis of fish supplemented with 500 mg of AP/kg of diet (Table 2; Figures 2B and 2D). The highest collagen layer was observed in fish submitted to the treatment of 1000 mg of AP/kg of diet. However, this layer was decreased in fish submitted to the 2500 mg of AP/kg of diet. These layers showed thickness and low collagen production in the fish without supplementation (Figure 2A and 2C).

The negative effect of high concentrations of vitamin C (2000 and 2500 mg AP/kg of diet) was observed for the first time in surubim juveniles.

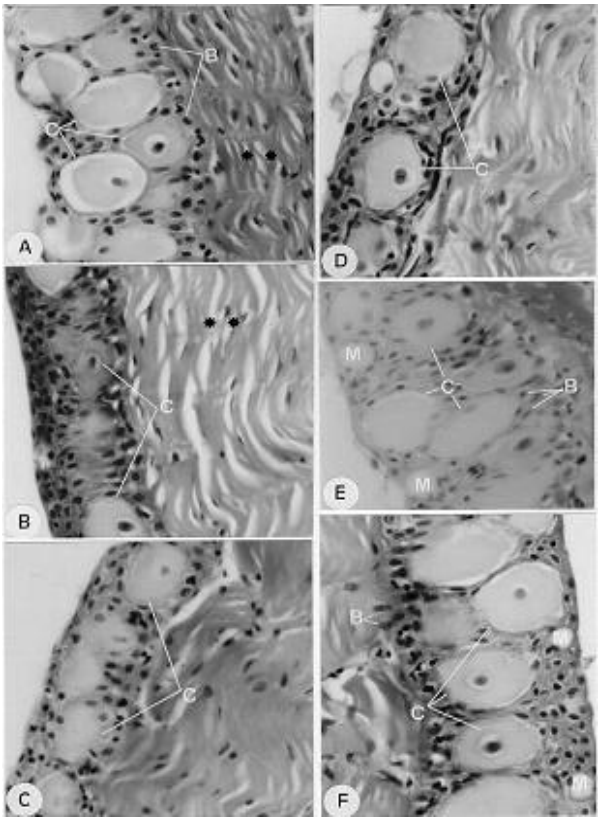

Figure 1. Micrograph of surubim (Pseudoplatystoma coruscans) skin. A: Detail of the basal cells (B) and club cells (C) in different levels (cellular disorganization), collagen layer in dermis (2 rosettes). Treatment of 0 mg of AP/kg diet. HE, 400x. B: Details of club cells (C) organization in the epidermis, dermis (2 asterisk). Treatment 500 mg of AP /kg diet. HE, 400x. C: Details of club cells (C) organization in the epidermis. Treatment 1000 mg of AP /kg diet. HE, 400x. D: Details of club cells (C) organization in the epidermis in treatment 1500 mg of AP /kg diet, HE 400x. E: Cellular disorganization in epidermis: club cells (C), basal cells (B), details of the mucus cells (M) in treatment 2000 mg of AP /kg diet. TM, 400x. F: Cellular disorganization in epidermis: club cells (C), basal cells (B), details of the mucus cells (M) in treatment 2500 mg of AP /kg diet. HE, 400x.

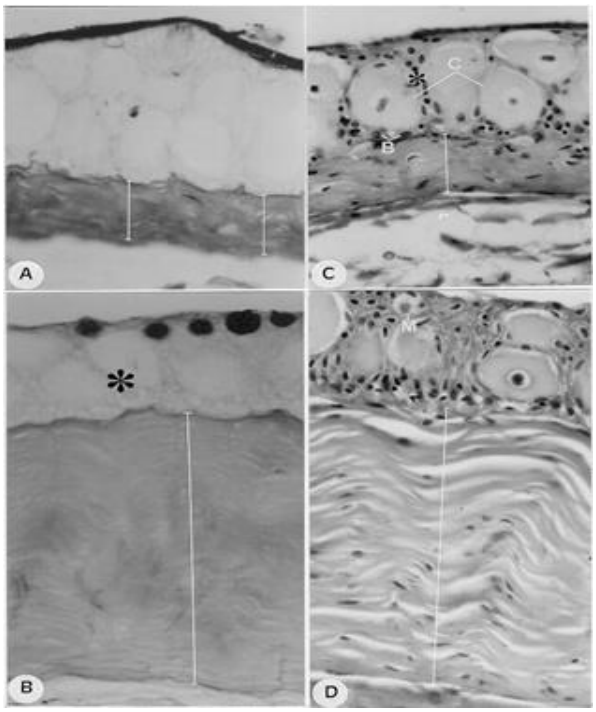

Figure 2. Skin stained micrograph of surubim (Pseudoplatystoma coruscans). A: Detail of thin collagen layer in dermis in treatment without vitamin C (0 mg of AP /kg diet)- PAS - 400x. B. Detail of collagen layer in dermis and mucus cells (asterisk) in the epidermis (asterisk) in treatment with 2000 mg AP/kg- PAS-400x. C: Epidermis (asterisk), evidencing the club cells (C), basal cells (B) and connective tissue, H-E- 400x. D: Detail of collagen in treatment with 2500 mg AP/kg. Epidermis: mucus cell (M). H-E, 400x.

Discussion

The monitored water parameters did not influence the fish maintenance: an average of 5.5 ± 1.35 mg of O_2/L dissolved oxygen and 29 ± 1.5 ºC temperature (Baldisseroto, 2002).

Morphology alterations were not observed in the surubim. The observed dermis *stratum compactum* formed by collagen fibers running parallel to the skin surface is similar to that reported by Genten et al. (2009). Wahli et al. (2003) did not observe any relationship between the epidermis and the use of vitamin C. However, they reported that the presence of vitamin C acts directly in the formation of basal epidermis cells and the process of healing injuries. Another study observed cellular disarrangement in the epidermis of *Cichlasoma urophthalmus* fed diets without vitamin C and fed with the minimum of 40 and 78 mg of ascorbic acid/kg of diet (Martinez and Richards, 1991). The observed similar clinical signs of cellular disarrangement (basal cells) in trout epidermis was related to vitamin A deficiency (Hibyia, 1982).

The intense collagen synthesis observed in fish under treatment is supported by the fact that collagen fibers are produced from proline hydroxylation in the presence of vitamin C to form a pro-collagen structure. Collagen fibers are flexible, have great tensile force, and are secreted by fibroblasts, which are the active cells of the *stratum compactum* in the dermis (Cavichiolo et al., 2002; Junqueira and Carneiro, 2004).

Therefore, the results show that the clinical signs observed in fish submitted to high levels of vitamin C are similar to those observed in fish with vitamin C deficiency. The effectiveness of megadoses reported in the literature is controversial. In many species, high levels of vitamin C do not produce beneficial results. Verlhac et al. (1994) observed an increased phagocytic activity in macrophages and activation of the complement system in trout fed with high doses of vitamin C. Abreu and Urbinati (2006) did not observe a reduction in the effects of the stress from air exposure in matrinxã, *Brycon amazonicus*, fed with 800 mg of ascorbic acid/kg of diet supplementation. However, Affonso et al. (2007) recommended the use of 800 mg of ascorbic acid/kg of diet for some species based on the observation of a high number of leukocytes and plasma protein in fish submitted to this supplementation level.

This controversy, in many cases, is justified by the age, reproduction pattern, management, or stress in fish. In the present study, the surubim skin showed morphological alteration at the 2500 mg/kg of diet supplementation suggesting a situation of hypervitaminosis, which thus indicates that this concentration should not be used. Observing the data reported by other authors allows the identification of other clinical signs associated with hypervitaminosis involving vitamin C such as a decrease in the level of raw protein in the carcass, weight gain, rate of specific growth, and RNA/DNA ratio and an increase in mortality rate as a consequence of *Aeromonas hydrophila*. Decreased phagocytosis, serine protein, lymphocytes, and lipophilic activity is observed in blood cells; however, there is an increase of thrombocytes and neutrophils (Misra, 2007; Tewary and Patra, 2008; Darias et al., 2011).

The excessive use of this vitamin leads to harmful alterations in the surubim, affecting the growth and increasing parasite infections as previously reported (Fujimoto and Carneiro, 2001). The observed increased number of monogenetic parasites in surubim fed without supplementation or supplemented with 2500 mg/kg of diet demonstrate these harmful alterations. Fin erosion and bone deformities have been observed in surubim fed without vitamin C supplementation (Fujimoto and Carneiro, 2001), which was probably caused by low collagen fiber production and consequently poor skin and bone formation.

Conclusion

Hypervitaminosis C occurs in spotted surubim when fed with mega doses of this vitamin. Decreased epidermis height, changes in club cells, and decreased collagen synthesis can be recognized as new clinical signs of hypervitaminosis C in fish.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Committee

The research project was approved by the ethics committee of UNIGRAN, under number 261/11.

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