FULL ARTICLE

ISSN Online 1678-4456

Effects of dietary vitamin E on the wound healing induced in *Oreochromis niloticus*

Efeitos da suplementação com vitamina E na cicatrização de feridas induzidas em Oreochromis niloticus

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ABSTRACT

In the present study, the effect of vitamin E supplementation 450 mg/kg diet was appraised in the process of induced wound healing in Nile tilapias *Oreochromis niloticus*. Fish were distributed into 18 tanks (10 fish each). Nine tanks were fed the non-supplemented diet and the other 9 tanks were fed 450 mg vitamin E for 60 days. Subsequently, the fish were anesthetized and the epidermis and dermis were surgically removed. The rate of cicatricial retraction and appearance of the wounds, and the histomorphometry of mucous cells, chromatophores, revascularization, inflammatory cells, presence of fibroblasts, collagen fibers, and scales were checked after 3-, 7-, 14-, 21-, and 28-days post-wounding. The retraction rate of the wound was significantly higher in the supplemented fish. The higher concentrations of inflammatory cells, mucous cells, and chromatophores, as well as the production and organization of collagen fibers, resulted in a higher retraction rate. We concluded that a dietary supplementation diet improves specific aspects of the cutaneous healing process in Nile tilapia fish.

Keywords: Alpha-tocopherol. Inflammation. Wound-healing. Nile tilapia.

RESUMO

No presente estudo, o efeito da suplementação com vitamina E de 450 mg / kg de dieta foi avaliado no processo de cicatrização induzida de feridas em tilápias do Nilo, *Oreochromis niloticus*. Os peixes foram distribuídos em 18 tanques (N=10), sendo 9 tanques com dieta não suplementada e os outros 9 tanques suplementados com 450 mg de vitamina E por 60 dias. Posteriormente, os peixes foram anestesiados e a epiderme e derme foram removidas cirurgicamente. Nos tempos pré-determinado de 3, 7, 14, 21 e 28 dias após a ferida foi analisado a taxa de retração cicatricial, a aparência das feridas e a histomorfometria das células mucosas, dos cromatóforos, das células inflamatórias, a revascularização, a presença de fibroblastos, de fibras de colágeno e escamas. A taxa de retração da ferida foi significativamente maior nos peixes suplementados. As maiores concentrações de células inflamatórias, mucosas e cromatóforos, bem como a produção e organização das fibras de colágeno, resultaram em uma maior taxa de retração. Concluímos que a dieta de suplementação melhora aspectos específicos do processo de cicatrização cutânea em peixes de tilápia do Nilo.

Palavras-chave: Alfa-tocoferol. Inflamação. Cicatrização. Tilápia do Nilo.

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Received: February 22, 2021
Approved: July 21, 2021

How to cite: Iwashita MKP, Engracia Filho JR, Claudiano GS, Aguinaga JY, Ozório ROA, Moraes JRE. Effects of dietary vitamin E on the wound healing induced in *Oreochromis niloticus* J Vet Res Anim Sci. 2021;58:e182254. https://doi.org/10.11606/issn.1678-4456.bjvras.2021.182254

Introduction

The use of immunostimulants in the feed helps to improve the organism's resistance to stress and, consequently, to disease. It has been reported that the rate of infection is associated with the defense response in several fish species (Castro et al., 2014; Martins et al., 2008; Reque et al., 2010; Sakabe et al., 2013). Researchers have favored the use of fish models to evaluate the immunostimulatory effects of dietary micronutrients against inflammatory disorders (Garcia et al., 2007; Moraes & Moraes, 2009; Salvador et al., 2013).

Vitamin E is an important nutrient that acts as a lipid-soluble antioxidant and immunostimulant that protects cell membranes and lipoproteins against oxidation and is also an essential dietary nutrient in most fishes (Bai & Lee, 1998; Montero et al., 1999). Wise et al. (1993) showed that channel catfish (*Ictalurus punctatus*) fed high doses of vitamin E presented increased leucocyte phagocytic activity and superoxide anion production. Similarly, Ortuño et al. (2001) reported that gilthead seabream (*Sparus aurata*) fed vitamin E-supplemented diet exhibited increased complement and phagocytic activities. Belo et al. (2005) showed that vitamin E may contribute to the efficiency of the fish inflammatory response by increasing macrophage recruitment and giant cell formation against foreign bodies. Furthermore, vitamin E appears to act on the stress response in pacu fish by preventing stress-related immunosuppression (Belo et al., 2005).

It has been established that the cells involved in the inflammatory response in fish are predominantly thrombocytes, lymphocytes, and macrophages (Claudiano et al., 2013; Martins et al., 2008, 2009; Reque et al., 2010). After the removal of injured tissue, the process of re-epithelialization occurs through the proliferation of granulation tissue, the reorganization of dermal connective tissue, and the formation of collagen fibers (Eckhoff et al., 1998; Iger & Abraham, 1990; Jorge et al., 2008; Moraes et al., 2003). This study aimed to evaluate the effect of dietary vitamin E supplementation in the induced tissue repair in Nile tilapia.

Materials and Methods

A total of 180 juvenile Nile tilapia (30 ± 2.5g) were distributed in 18 tanks of 250 L each and fed basal formulation of the experimental diets in Nile tilapias for 10 days, ingredients: soybean meal (43%), cornmeal (22.3%), wheat bran (17%), rice bran (10%), yeast (4%), L-lysine (0.2%); DL-methionine (0.4%), bicalcic phosphate (1%), limestone (1%) and premix composed of vitamins and mineral supplied by the diet - FRI RIBE (Vit E was added according to the treatment; 450 mg tocopherol acetate.kg^{-1} of diet – 0.5%) (Ethics Committee Approval No. 02.433/10).

After that, 9 tanks were used as control groups using a vitamin E-free diet (n = 90) and in the remaining tanks, fish were supplemented with 450 mg of vitamin E / kg^{-1} of food (Belo et al., 2005) for 60 days (Garcia et al., 2011). Fish diets were prepared and stored in dark plastic bags at -4 ºC until use. The source of vitamin E (Rovimix E-50 Adsorbato, Roche®) was incorporated into the experimental diet according to Castro et al. (2014). All fish in the study were subjected to biometric evaluation every two weeks, considering total length and weight.

Water quality parameters were measured weekly. The physical-chemical parameters of the water remained constant throughout the experiment: temperature (30.20 ± 0.8 ºC), dissolved oxygen (7.96 ± 2.48 mg L^{-1}), pH (7.96 ± 0.28), electrical conductivity (179 ± 69 µS cm^{-1}), total dissolved solids (89 ± 34 ppm), and salinity (0.08 ± 0.01 ppm).

After 60 days of supplementation, the induction of surgical wounds was carried out following Bortoluzzi et al. (2017). First, fish were anesthetized in an aqueous solution of benzocaine 1:20.000 (Cosenza et al., 2014). The area of the wound represented approximately 3% of the body surface of the fish (Moraes et al., 2003). The vertices of the experimental wounds (2.0 cm width x 1.0 cm length x 0.25 cm depth) were outlined using a sterile plastic template on the left lateral region of the fish. A full-thickness flap of skin was removed by cutting the epidermis and dermis with a scalpel. When necessary, hemorrhage was controlled by applying pressure with the sterile cotton gauze. No fish died due to the experimental wounding.

Samples were taken at 1-, 3-, 7-, 14-, 21-, and 28-days post-injury from 15 control and 15 supplemented fishes per

experimental time (adapted from Bortoluzzi et al., 2017). The samples were processed using histochemical methods.

The macroscopic evaluation was performed by tracing the wounded area in a plastic film using a planimeter and calculating the degree of retraction. Thereafter, the index of retraction (IR, %) of the wound was determined by the equation (Araújo et al., 2010): RI = (initial area of the wound - final area of the wound) × 100/initial area of the wound

Skin samples (wound, adjacent edges, and muscle layers) were removed for histological analyses. Half of each sample was fixed in Bouin solution, paraffin-embedded, sectioned, and stained with Sirius-red to identify collagen fibers (Junqueira et al., 1982). The remained portion was fixed in 10% buffered formalin, embedded in resin, sectioned, and stained with toluidine blue (1%) (Hopwood, 1990; Santos & Oliveira, 2007).

Tissue evaluations were performed in a binocular light microscope coupled to a digital camera (Nikon E200, Nikon, Tokyo, Japan, and Olympus DP 72, with Cell Sens v.1.5 imaging software). All images were treated with an automatic contrast option (removing shadows with the enhancement of the image and mapping of the lighter and darker pixels remaining in the image). Before counting, the cell types and distribution was analyzed (Bezerril et al., 2020). To quantify the frequency, five fields were randomly selected per section regarding the degree of epithelial hyperplasia, the number of mucous and chromatophores cells, neovascularization, presence of inflammatory cells, amount of collagen, and scale formation in each group and time (Bortoluzzi et al., 2017), using a 40x objective. Results were expressed as the percentage of labeled cells and/or the number of cells (Bezerril et al., 2020).

All results of biometrics, healing area, and histomorphometry were submitted to analysis of variance in a completely randomized design (Kolmogorov-Smirnov, Anderson-Darling, Shapiro-Wilk, and Watson) and comparison means through Tukey test ($p < 0.05$). The retraction index of cicatrization was submitted to the Chi-square test. The cell counts means were compared among the time with one-way ANOVA type III, followed by Tukey´s test. The mean cell counts between control and treated groups were compared with an independent t-test, considering non-homocedasticity cases. All statistical analyses were performed using the SAS program (SAS version 9.1).

Results and Discussion

There were no differences in the biometric values between the control and supplemented groups. Macroscopically, the skin wounds in the control group remained unchanged throughout the observation period. In the supplemented group, the wounds were initially rectangular and then became circular. Morphometric analysis showed no variation in the shape and size of the healing area before day 7, regardless of the dietary treatment. However, after day 14, fish fed vitamin E-supplemented diet showed differences in the shape and size of the healing area.

The color of the wounds was also evaluated over time. The wounds of the control group ranged from pinkish (day 3) to red (day 7) and gradually become grey by day 28. The wounds in the supplemented group were more reddish on the third day with abundant vasculature and diffuse in the central area. The color then progressed towards the color of normal skin (silver) on day 14. Before the end of the experiment, the wound of supplemented animals was covered with silver skin, typical of Nile tilapia.

There was no difference between the rate of wound retraction in animals supplemented with vitamin E and the control group at the end of the wound healing evaluation period (Table 1).

The comparison of the index of wound retraction between the control and supplemented groups among the times showed differences in the average of scarring areas ($p < 0.01$; Figure 1). Comparing the rates of cicatricial retraction, we found that the group supplemented with vitamin E presented a higher rate, confirming the hypothesis that dietary vitamin E supplementation provides a protective mechanical barrier, which is essential for protecting newly regenerated tissue in fish (Silva et al., 2005).

In this study, signs of re-epithelialization were evident in both groups after 3-days post-injury. This corroborates

Table 1 – Effects of dietary vitamin E supplementation on retraction index (RI) of tissue repair in Nile tilapia, after the induction of the healing process

Treatments	RI
Control	22.95%
Vitamin E	24.04%
p-value	0.9712

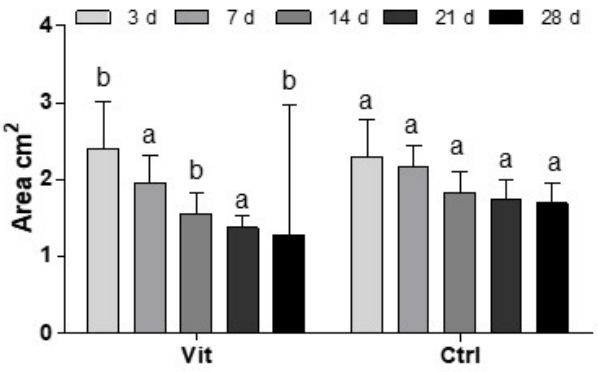

Figure 1 – Effects of dietary vitamin E supplementation on scar area (cm^2) of wounds surgically induced in Nile tilapia. In each column (n=18 / $p < 0.05$), means followed by same capital letter do not differ. In each column, the same lowercase letters do not differ.

results obtained by Moraes et al. (2003) in *P. mesopotamicus* supplemented with 500 mg of vitamin C kg^{-1} food and Wahli et al. (2003) in rainbow trout supplemented with different concentrations of vitamin C. A comparison of the rate of re-epithelialization between control and supplemented groups showed no differences, likely because vitamin E does not interfere in this process, at least at the dose used in this experiment. However, the fact that re-epithelialization occurred quickly supports its role as a barrier to opportunistic infections and osmotic imbalance (Roubal & Bullock, 1988; Silva et al., 2004).

The effects of dietary vitamin E supplementation on mucous cell count, chromatophore count, neovascularization, inflammatory cell migration, and scale formation are listed in Table 2. The number of mucous cells increased gradually ($p< 0.01$) over the post-injury stages, mainly between groups on days 21 and 28 (Table 2, Figure 2 S). These suggest that vitamin E aids the inflammatory process in response to stress stimuli, significantly increasing the number of mucous cells over time (Ortuño et al., 2001; Belo et al., 2005; Martins et al., 2008). The mucus produced by these cells is rich in lysozyme and antibodies and plays an important role in the primary defense mechanisms against pathogens. It seems that vitamin E may increase the mechanism of protection attributed to these cells (Noga, 1996). Similar results were also observed in pacu (Petric et al., 2003) and rainbow trout (Wahli et al., 2003) supplemented with vitamin C.

The chromatophore (CRM) count was statistically different between control and supplemented groups on days 7, 14, 21, and 28. Higher values were recorded for the supplemented group on days 3, 14, and 28 (Table 2). The CRM counts were measured starting on the first sampling day and increased progressively and more rapidly in the supplemented group compared to the control. A higher quantity of pigment was noticed in the wound on day 14, which confirms the histological results (Figure 2 S28). Such an increase in pigment would be responsible for the change in the color of the wound from reddish to grey, since there is an intense replacement of CRM and melanocytes in the epidermis and, subsequently, in the dermis (Figure 2 S14 and S28; Figure 3F).

The same phenomenon was also described in *Notothenia Coriiceps* (Silva et al., 2004, 2005). Anderson & Roberts (1975) reported the presence of CRM in Atlantic salmon only from day 4 post-injury and onwards. Furthermore, the presence of CRM is related to the immune and inflammatory responses of chronic lesions[2]. In the current study, the increase in pigment concentration may be related to vitamin E increasing the migration of these cells to the point of injury, further potentiating the defense mechanism during the inflammatory process and healing. This result confirms those observed by Belo et al. (2005) in their studies in *Piaractus mesopotamicus* fed for 60 days with a supplemented diet with vitamin E (450 mg/kg^{-1}), evidencing that the vitamin induces better inflammatory response with consequent healing in fish of commercial interest.

The neovascularization (NEO) count was statistically different between the two groups ($p< 0.01$, Table 2). The NEO count varied between treatments on days 3 and 7, wherein the values were higher in the supplemented group (Figure 3D). Good blood perfusion is essential for the healing of tissues (Silva et al., 2004). The vascular neoformation was maximal on days 7 and 14 for the supplemented and control groups, respectively, and gradually decreased thereafter (Table 2 and Figure 3D). These data confirm those described by other studies (Moraes et al., 2003; Wahli et al., 2003) that tested the effects of dietary vitamin C supplementation on the healing process of induced skin injuries in fish. The analyzed data lead us to conclude that vitamin E supplementation accelerated the rate of angiogenesis at the local site of injury and helped decrease the chances of secondary infections in fish farms.

Table 2 – Effects of dietary vitamin E supplementation on the counts of mucous cells (MC), chromatophores (CRM), neovascularization (NEO), inflammatory cells (IC), and scales (ESC) of the skin of Nile tilapia, at 3, 7, 14, 21, and 28 days (n=18) after the induction of the healing process

Cell counts										
Days of evaluation	**3**		**7**		**14**		**21**		**28**	
Treatments	**Cntrl.**	**Vit.**	**Cntrl.**	**Vit.**	**Cntrl.**	**Vit.**	**Cntrl.**	**Vit.**	**Cntrl.**	**Vit.**
MC	1.87^{Ea}	1.67^{Ea}	2.71^{Da}	2.87^{Da}	3.32^{Ca}	3.55^{Ca}	4.06^{Bb}	4.92^{Aa}	5.22^{Aa}	4.13^{Bb}
SD	0.49	0.27	0.29	0.66	0.35	0.71	1.21	0.91	1.27	1.18
CRM	2.12^{Ea}	2.12^{Ea}	2.86^{Db}	3.82^{Da}	3.76^{Cb}	4.38^{Ca}	8.76^{Aa}	6.34^{Bb}	6.17^{Bb}	6.79^{Aa}
SD	0.41	1.01	1.89	1.01	1.74	1.08	3.37	3.51	3.14	3.64
NEO	2.63^{Eb}	3.51^{Ba}	3.10^{Bb}	3.68^{Aa}	3.25^{Aa}	3.08^{Ca}	2.86^{Ca}	2.61^{Da}	2.64^{Da}	2.27^{Ea}
SD	0.42	0.33	1.89	0.47	1.74	0.35	0.30	0.34	0.31	0.31
IC	2.09^{Db}	3.29^{Aa}	2.61^{Ba}	2.53^{Ca}	2.79^{Aa}	2.69^{Ba}	2.27^{Ca}	1.87^{Eb}	2.07^{Eb}	2.42^{Da}
SD	0.49	0.51	0.05	0.29	0.03	0.27	0.62	0.17	0.20	0.19
ESC	1.03^{Aa}	1.01^{Aa}	1.08^{Aa}	1.07^{Aa}	1.08^{Aa}	1.03^{Aa}	1.07^{Aa}	1.03^{Aa}	1.00^{Aa}	1.00^{Aa}
SD	0.05	0.03	0.05	0.07	0.03	0.06	0.08	0.06	0.04	0.06

In each row, means followed by the same capital letter do not differ among times (ANOVA+Tukey). Same lowercase letters do not differ between control and treated groups (t-test). (n=18) $p < 0.05$. SD - Standard deviation.

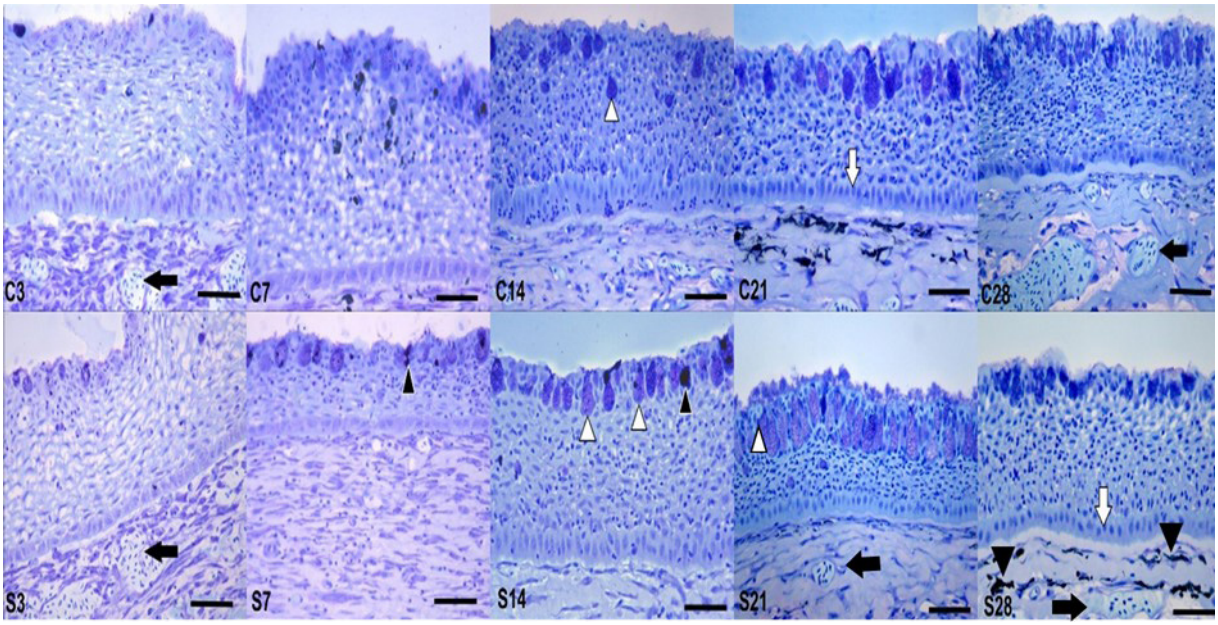

Figure 2 – Nile tilapia skin epithelium of control (C) and supplemented group with 450 mg vitamin E kg^{-1} diet (S) after 3, 7, 14, 21, and 28 days post-injury (DPI). Basal layer (white arrows). Note the increasing number of mucous cells (white arrowheads) post-injury, evidenced after 14 DPI (S14); Early neovascularization in the supplemented group (black arrows). Chromatophores (black arrowheads) increased progressively and more rapidly in the supplemented group (S28). Toluidine Blue staining. Bar = 100µm.

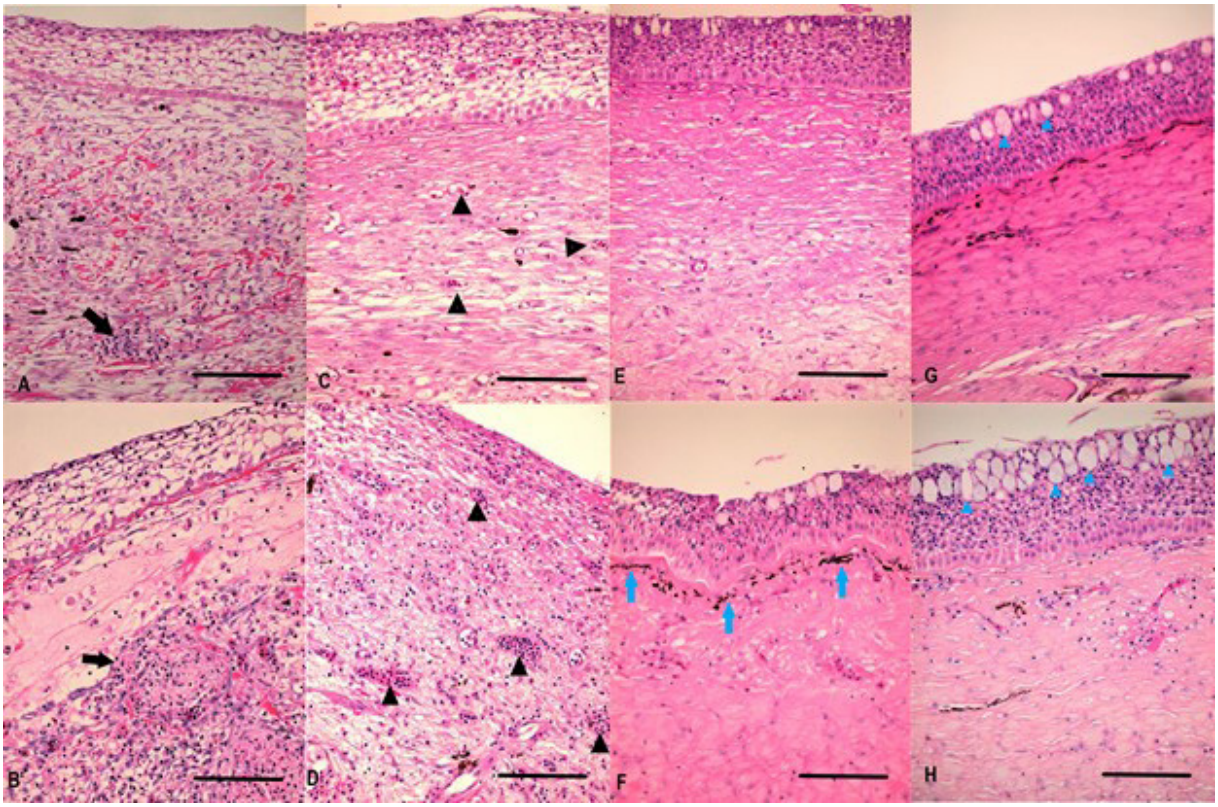

Figure 3 – Nile tilapia skin epithelium of control (A, C, E, and G) and supplemented group with 450 mg vitamin E kg-1 diet (B, D, F, and H). Skin from the control group (A) showed fewer inflammatory cells (black arrows) in comparison to the supplemented group (B) on day 3 post-injury. On day 7, neovascularization was higher in supplemented group (D) than the control group, showing more newly formed blood vessels (black arrowheads). Chromatophore (blue arrows) count was higher in the supplemented group (F) than in control (E) on days 7 and 14 post-injury. Note the higher number of mucous cells (blue arrowheads) in supplemented group (H) than the control group (G) on days 21 and 28. Hematoxylin and Eosin. Bar = 100µm.

The inflammatory cell (IC) count was significantly different between the two groups on days 3, 21, and 28, thus indicating that vitamin E supplementation promotes the migration of inflammatory cells to the focus of a lesion

(Table 2 and Figure 3B). The results for the increase in IC migration agree with Belo et al. (2005), who described the effect of vitamin E in chronic inflammation induced by the implantation of subcutaneous glass blades in the pacus. Thus, we can say that vitamin E supplementation accelerated the migration of these cells as part of the inflammatory defense mechanism. Conversely, no significant differences were found for the formation of scales (ESC) between groups (Table 2 and Figure 3).

Collagen fibers were more organized in the vitamin E-supplemented diets than in the control group (Figure 4). More fibers were arranged parallel to the surface of the skin and with a greater degree of organization. It was possible to verify a larger amount of collagen fibers in the control group, starting on day 3, but there was still an overall lower degree of the organization compared to the supplemented group. These observations suggest that vitamin E affects the production of collagen, which may be an additional mechanism whereby vitamin E increases the process of tissue repair in tilapia. In studies with mammals, Lall & Lewis-McCrea (2007) observed that supplementation with vitamin E decreased the resorption of bone matrix and stimulated its proliferation, through the inhibition of cytokines IL1 and IL6. These data corroborate with those found by Moraes et al. (2003) who studied healing in pacus supplemented with vitamin C. In the same line of research, Jauncey et al. (1985) observed that fish fed low levels of vitamin C showed an impairment of tissue repair by interfering with collagen synthesis.

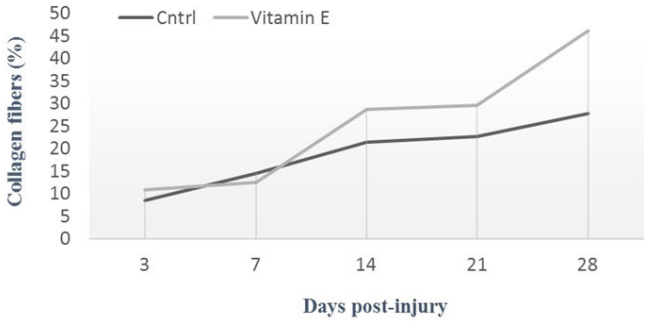

Figure 4 – The wound healing process in Nile tilapias supplemented with vitamin E. Effects of dietary vitamin E supplementation on the collagen fibers (%) of the skin of Nile tilapia, after induction of the healing process.

The obtained conclusion was that dietary supplementation with 450 mg vitamin E kg^{-1} diet significantly increases the number of mucous cells, chromatophore count, neovascularization, and inflammatory cell migration to the focus of a lesion. Also, the dietary supplementation has a positive effect on the production of collagen improving the cutaneous healing process in Nile tilapia fish. It is an important alternative to reduce injuries caused during animal management and improving their welfare.

Conflict of interest

There is no conflict of interest between authors.

Ethics Statement

Ethics Committee Approval No. 02.433/10

Acknowledgements

The authors thanks the Research Support Foundation of the State of São Paulo (grant nº 2007/52523-6), to CNPq (National Counsel of Technological and Scientific Development) for financial support to F.R. Moraes and J.R.E. Moraes. and Coordination for the Improvement of Higher Education Personnel (CAPES) - Finance Code 001. *In Memoriam*: A Great Friend and Mentor, Professor Flávio Ruas de Moraes.

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Financial Support: The authors thanks the Research Support Foundation of the State of São Paulo (grant nº 2007/52523-6), to CNPq (National Counsel of Technological and Scientific Development) for financial support to F.R. Moraes and J.R.E. Moraes. and Coordination for the Improvement of Higher Education Personnel (CAPES) - Finance Code 001.