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Short communication

β-Glucan improves wound healing in silver catfish (*Rhamdia quelen*)

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ABSTRACT

The immune modulating activity of β-glucan on aquatic species has been a matter of intense investigation. Here, we aimed to investigate the effect of β-glucan on wound healing of silver catfish, a Neotropical South American scale-free fish. Small sections of skin and muscle (3 mm in diameter) were removed and fish were bathed daily with β-glucan (0.1% and 0.5%) up to 28 days when cicatrization was complete. A group of fish similarly injured and non-exposed to β-glucan was used as control. Wound closure and healing was monitored visually and by histopathological analysis. In fish bathed with 0.5% β-glucan we found reduced blood cortisol levels at day one post-wounding and, by day 7 post wounding, the deposition of granulation tissue was higher compared to non-exposed fish. In addition, from day 7 forward, wound size was significantly lower in fish bathed with 0.5% β-glucan. Histopathological analysis of the wounded site indicated a thin layer of immature epidermal cells at day one post wounding. A discrete inflammation with mixed inflammatory cell infiltrate was observed on wounded muscle and was lower by day 7 post wounding on fish bathed with 0.5% β-glucan. By day 14 post wounding, the deposition of collagen fibers and the presence of fibroblast and new muscle fibers were higher in fish exposed to 0.5% β-glucan, and dermis restoration was complete. Thus, our results indicate that in silver catfish wound healing occurs rapidly and improves greatly by daily bathing with β-glucan.

1. Introduction

Skin is the second largest tissue of vertebrates and a main constituent of the innate immune system [1]. In certain fish species, scales and mucous provide additional barrier to prevent pathogen access through the skin to subcutaneous tissues and vascular system [2,3]. Thus, a well-structured physical barrier is fundamental to aquatic organisms dwelling on an environment usually populated with all sort of microorganism able to cause a variety of diseases [4]. Skin wounds in fish are caused mainly by physical trauma, ectoparasites and Gram negative bacteria [5] and represents an opportunity to microorganism colonization and secondary infection. Thus, promptly skin and underlying tissue repair should occur rapidly to reduce infection opportunities [6].

The healing process aims to repair tissue damage and initiates with an inflammation reaction followed by temporally overlapping step of new tissue formation and tissue remodeling [6,7]. This complex set of well-organized immune-related reactions might be influenced by immune-suppressive molecules such as cortisol or enhanced by immune-stimulating compounds [7]. Indeed, in mammals, the wound healing process has been improved by the use of β-glucan [8,9], a well-known pathogen-associated molecular pattern (PAMP) molecule ubiquitously found in nature but obtained mostly from yeast [10] for commercial purposes. In fish, the immunomodulatory effect of β-glucan on wound healing has been evaluated in common carp, *Cyprinus carpio* L. [11]. and rainbow trout *Onchorhyncus mykiss* [12], two worldwide cultured scaled fish. In common carp exposed to β-glucan there was an upregulation on the expression of IL-1β and IL-6 in the skin and IL-8 on muscle at three days after wounding compared to non-treated wounded fish [11]; those cytokines are central to inflammatory reaction and could account for by improving tissue healing in this species. In contrast, a similar study conducted with rainbow trout found no effect of β-glucan bathing on wound healing which [12], in turn, took much longer to completion.

Skin wounds are also commonly observed in brood stock silver catfish (*Rhamdia quelen*), a bagrid Neotropical fish species commonly found in Central and South America [13]. In previous studies we demonstrated that β-glucans used as adjuvant improves antibody production to a model antigen [14] and when added to the diet increases resistance to experimental *Aeromonas hydrophila* challenge [15]. Here,

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we investigated the effect of β-glucan enriched daily water bath on wound healing by monitoring wound size, cortisol production and histopathological changes on wounded tissue. We found that tissue regeneration occurred rapidly and was improved by the use of β-glucan.

2. Material and methods

2.1. Fish

One hundred and fourteen juvenile mixed sex silver catfish (30 ± 5,5 g) were equally divided into three indoor tanks (38 fish/tank, 500 L) containing running water (23-25 °C) from a natural pond and acclimatized for 7 days. Water parameters of dissolved oxygen (5.5–7.0 mg/L), pH (7.1 ± 0.3), hardness and alkalinity (45 ± 5 mg $CaCO_3$/L) and total ammonia (< 0.7 mg/L) were within accepted range for the species. Prior to and during the experiments fish were fed commercial pelleted food (crude protein 30%; fiber 3%; fat 8%; and ash 14%; Supra, Brazil). In addition, prior to each experiment, randomly selected fish were inspected externally for disease sign and had blood samples collected for bacterial cultivation, to ascertain they harbor no specific bacterial pathogens in their tissues. All sampling and inoculation procedures were carried out with anesthetized fish (Eugenol, 50 mg/L^{-1}).The study was carried out in accordance with the regulations of the National 495 Council for the Control of Animal Experimentation (CONCEA) and approved by the Institutional Ethical and Animal Welfare Committee (CEUA protocol number 042/2017).

2.2. Experimental design and sampling procedure

Fish wounding and exposure to β-glucan started at the same day. For wounding, fish were captured, anesthetized and then a tissue section (3 mm in diameter) of skin and muscle (5 mm in depth) was removed from the dorsal area close to the central fin, in both sides (1 punch in each side) using a biopsy punch. Water baths for fish treatment were prepared by stopping the water flux and adding of β-glucan to the tank water to reach 0.1% (0.1 mg/L) or 0.5% (0.5 mg/L). Fish were bathed daily for 1 h and then the water flux was opened. β-Glucan (Macro Gard, yeast, > 60% pure, Biorigin, Brazil) was prepared as previously described [11] and used throughout the experiment. A third control group had no β-glucan on the water. At days 1, 3, 7, 14, 21 and 28, 6 individuals from each tank were captured for wound analysis, blood and tissue sampling. To do that, silver catfish were anesthetized and an image of the wounded area was acquired using a regular HD camera. Fish were placed under artificial light and the camera was always kept exactly at the same distance from the fish (30 cm). The wound area on the image was manually outlined at the dermis edge and the number of pixels on the wounded open area was counted to estimate wound size. Wound size at day 1 post-wounding, in percentage (100%), was used to estimate the healing progress.

Then, blood samples were collected from the caudal vein and allowed to clot on ice to obtain serum for cortisol analysis using a commercial ELISA assay, as recommended by the manufacturers (EIAgen CORTISOL test; BioChem ImmunoSystems, Rome, Italy). To evaluate histopathological changes on wounded tissue, the previously wounded area and surrounding tissue were removed using a larger biopsy punch (6 mm in diameter) as demonstrated and described previously [11,12]. Biopsy samples were immediately fixed in 10% buffered-formalin for 24 h and then embedded in paraffin blocks. Thin sections (5 μm) were removed, fixed in glass slides and stained with hematoxylin and eosin (H&E) for histopathological analysis. Microscopic lesions were scored by number as normal tissue (0) or with mild (1 = ≤ 30%), moderate (2 = ≥ 30% but ≤ 60%) or accentuated/high (3 = ≥ 60%) changes, considering the parameter evaluated.

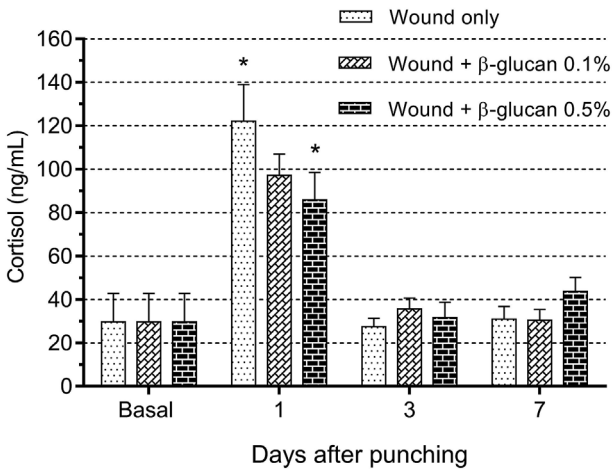

Fig. 1. Blood cortisol levels on wounded fish. Blood samples were taken prior to wounding (basal levels) and at the indicated days post wounding to determine cortisol concentration. The data was analyzed by Anova followed by Tukey's post-test and represents the mean ± SEM (n = 6). Significant differences between groups are indicated by asterisk ($p < 0.0405$).

2.3. Statistical analysis

The data were evaluated by the Shapiro-Wilk's test and found to have normal distribution. Differences amongst treatments were analyzed by Anova, followed by Tukey's post-test, as stated on figure legends, and plotted using GraphPadPrism Software v7 (GraphPad Software, Inc, USA). Results are reported as the mean ± standard error of the mean (SEM) and p values equal to or smaller than 0.05 were considered to be significant.

3. Results

A raise in cortisol production was observed only on day 1 post-wounding; the levels of cortisol were significantly lower ($p = 0.0405$) in fish bathed with 0.5% β-glucan (Fig. 1) compared to fish from control group. At days 3 and 7 post-wounding cortisol levels returned to basal levels.

To estimate wound regression, the size of the wounds was estimated as percentage from the original wound (day 1 post-wounding). Wounds reduced rapidly and by day 7 post-wounding there was a significant reduction ($p = 0.0156$) of the wound size in fish treated with 0.5% β-glucan (32.5% reduction) compared to wound size reduction (21.1%) on the skin of control fish (Fig. 2). By day 14 post wounding a 71.4% reduction on wound size was observed in fish treated with 0.5% β-glucan compared to 60.9% reduction on control group ($p = 0.0288$). Healing of skin was complete by day 21 post-wounding. Afterwards, visual differences within groups were noticed only on skin pigmentation that was still lower on the 0.1% β-glucan and control group up to 28 days post-wounding (Fig. 3) but considered normal on fish from the 0.5% β-glucan treated group.

The main histopathological changes on the skin and muscle during healing are described on Table 1. Differences between control and β-glucan treated groups are better noticed from day 7 post-wounding forward. By day 7, abundant granulation tissue is present on fish from the 0.5% β-glucan treated group. Fibroblast, collagen and new muscle cells are seen by day 14 on both β-glucan treated groups but not yet on control fish. In addition, young fibroblast cells are more prominent in the dermis of both β-glucan treated groups. By day 21 post-wounding, muscle fiber regeneration is high on fish bathed with 0.5% β-glucan compared to the other groups, and fibrosis and inflammation is observed only on fish from the control and 0.1% β-glucan treated group. Dermis regeneration is high on the 0.5% β-glucan exposed fish by day

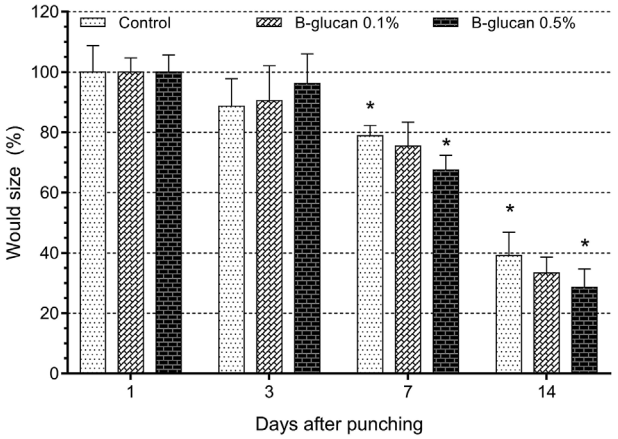

Fig. 2. Wound healing kinetics. Regression of wound size was estimated as the percentage of the original size of the wound in each group. The data was analyzed by Anova followed by Tukey's post-test and represents the mean ± SEM (n = 6). Significant differences between groups are indicated by asterisk and were observed at day 7 (p = 0.0156) and 14 (p = 0.0288) post wounding between control and the 0.5% β-glucan treated group.

28 post-wounding and skin layers are complete in both β-glucan exposed group, whereas in the control group there is still signs of muscle fiber regeneration, fibrosis and inflammation. The histopathological aspect of the wounded site and subsequent regeneration is depicted on Fig. 4; by day 28 post-wounding, on 0.5% β-glucan treated fish, no fibrosis or inflammation could be observed on muscle and intense regeneration and skin layer were observed on dermis. Epidermis aspect was normal in fish from all groups.

4. Discussion

Wound healing is an essential physiological process of vertebrates that aims to restore the original tissue. It comprises temporally overlapping events that initiates with an inflammatory reaction immediately after tissue damage, followed by cell proliferation and tissue remodeling [2,6,7]. There are several factors that might perturb signaling pathways and delay or accelerate wound healing, and this has been a matter of much investigation [7]. Although skin lesions and wounds in fish might not be as common as in terrestrial animals, they might be even more difficult to detect and to treat [2,3]. Nonetheless,

Table 1
Grading and main histopathologic findings at the wounded site.

Day	Tissue	Main findings on the wounded site	Treatment		
			Wound only	Wound + β glucan 0.1%	Wound + β glucan 0.5%
1	Muscle	Necrosis	3	3	3
		Edema	3	3	3
		Fibrin	3	2	2
		Hemorrhage	3	2 to 3	2 to 3
		Inflammation	2[a]	2[a]	2[a]
7	Muscle	Necrosis	1 to 2	1	1
		Granulation tissue	1	1	3
		Inflammation	2[a]	2	1
	Epidermis		Immature[1,a]	Immature	0
14	Muscle	Granulation tissue	2	–	–
		Inflammation	1	1	1
		Fibroblast	–	2	2
		Collagen	–	2	2
		New muscle fibers	–	1 to 2	1 to 2
	Dermis	Young fibroblast	3	3	3
		Collagen	2	3	2 to 3
	Epidermis		Maturation 1-2	0	0
21	Muscle	Muscle fibers regeneration	1	2	3
		Fibrosis	2	–	–
		Inflammation	1[b]	1	–
	Dermis	Fibroblast	2	3	3
		Collagen	2	3	3
		Melanophors	1	2	2
		Skin layers	≤30%	≥60%	≥60%
	Epidermis		0	0	0
28	Muscle	Muscle fiber regeneration	1	2 to 3	3
		Fibrosis	1	1	–
		Inflammation	1	1	–
	Dermis	regeneration	2	2	3
		Fibroblast	1 to 2	1 to 2	1
		Skins layers	≥30 - ≤60%	≥60%	complete
	Epidermis		0	0	0

Grading: 0 = normal tissue; 1 = mild; 2 = moderate; 3 = accentuated/high.
[a] Mixed inflammatory infiltrate.
[b] Mononuclear infiltrate.

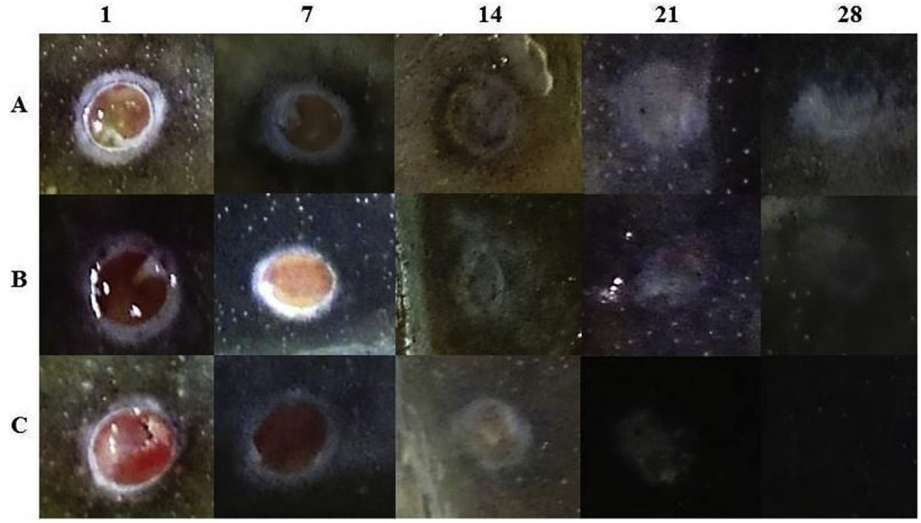

Fig. 3. Post wounding images from (A) non-exposed control fish and from (B) 0.1% and (C) 0.5% β-glucan exposed fish. Closure of the wounded site is apparent at day 7 forward in 0.5% β-glucan treated fish. In non-exposed control fish a hypopigmented area is noticed by day 28 post wounding.

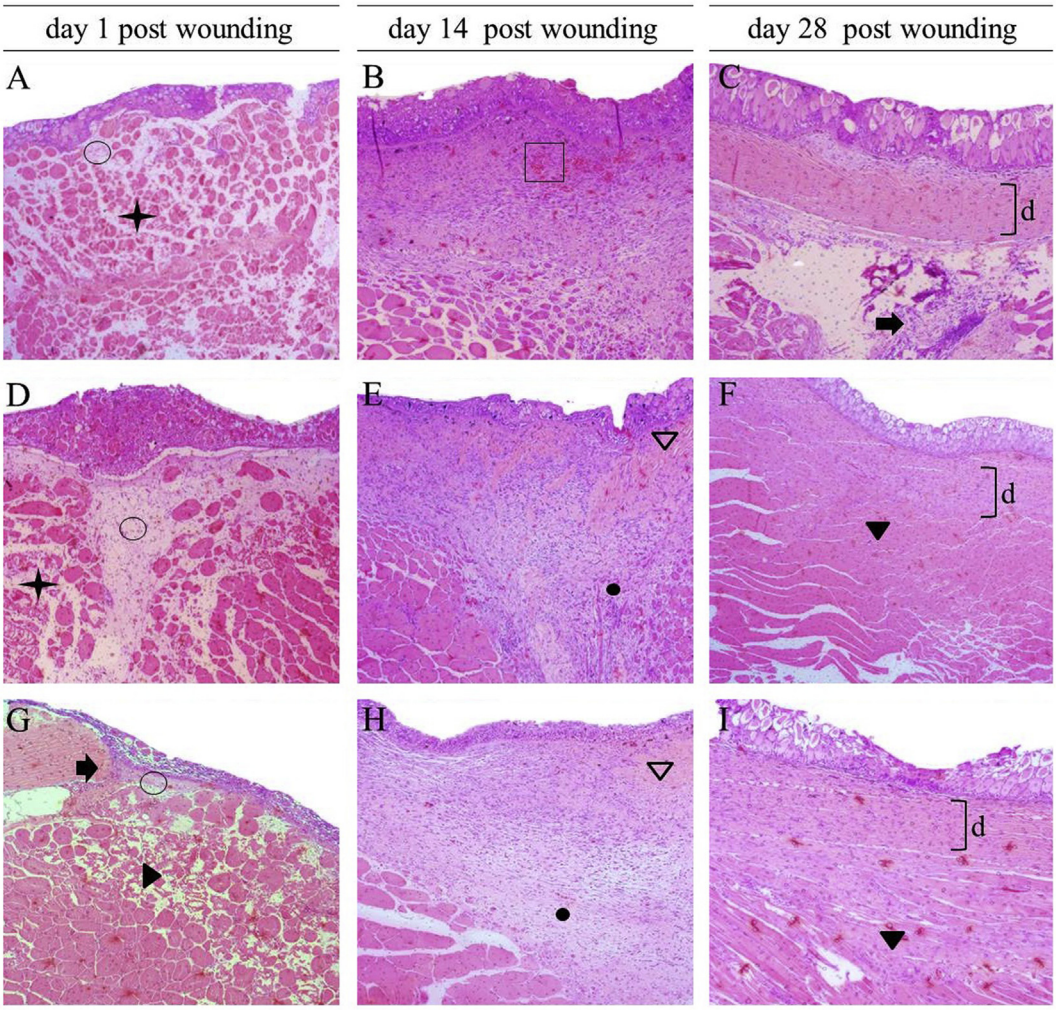

Fig. 4. Histopathologic analysis of the wounded site. Samples from non-exposed fish are indicated at the top: (A) large area of necrotic muscle fibers (star), covered by an immature thin epidermal layer, and interspersed with fibrin (open circle) within muscle fibers; in (B), there is neovascularization (open box) and (C) mixed inflammatory reaction (arrow) with a mature and intact dermis (]d). In the middle, samples from 0.1% β-glucan bathed fish: (D) absence of dermis, degeneration and necrosis of muscular tissue (star), and fibrin deposition (open circle); (E) New muscle fibers interspersed by collagen fibers and new fibroblast cells (closed circle); dermic tissue formation with the presence of collagen (open arrow head); (F) complete muscle fiber regeneration (closed arrow head) and dermis (]d). At the bottom, samples from fish exposed to 0.5% β-glucan: (G) the arrow indicates dermis interruption; remains of muscular fibers (arrow head) and fibrin (open circle) covered by a thin layer of immature epidermis are noticed; (H) development of new muscular fibers and regeneration of collagen fibers and young fibroblast cells (closed circle), intense collagen deposition (open arrow head), integral dermal tissue with a complete epidermal cell layer; (I) complete muscle fiber regeneration (arrow head) and dermis containing young fibroblast cells overlapped by a normal epidermal layer.

physical trauma, parasites and several bacteria are important causes of skin lesions which, once ruptured, represent an opportunity to secondary infection and access to subdermal tissues and vascular system.

Recently, the effect of a well-known pathogen-associated molecular pattern (PAMP) molecule (β-glucan, MacroGard®) has been used to evaluate wound healing in common carp and rainbow trout. Whereas β-glucan improved wound healing on common carp [11], no effect was observed in rainbow trout [12]. In our study, we used a non-scaled Neotropical skin fish to evaluate tissue repair and found that β-glucan bathing stimulated wound healing. Compared to common carp and rainbow trout, wound regression occurred more rapidly in silver catfish. In common carp and rainbow trout, a significant lesion was still present by day 14 and 100 post-wounding respectively [11,12], but in silver catfish wound regression was almost complete by day 14 and concluded by day 21 post wounding. At this day, in silver catfish bathed with 0.5% β-glucan, only a small hypopigmented area could be observed. And, in control fish, hypopigmented area was still observed by day 28 post-wounding. The differences observed on healing rate might be attributed to several factors such as fish species, the presence or absence of scales and water temperature; accordingly, rainbow trout was kept at 8.5 °C, common carp at 21 °C and silver catfish at 23 to 25 °C and this could certainly affect the rate of cell proliferation and cytokines secretion. Indeed, the expression of IL-1β mRNA, for instance, was markedly inhibited in trout leukocytes kept at low temperature [16] and much lower in the spleen of vaccinated rainbow trout kept at 5 °C compared to those kept at 15 and 25° [17] and this could most likely affect the production of IL-1β protein. IL-1β is central to inflammation [18], which is the first step of the healing process, and plays a major role on muscle catabolism to release amino acids for synthesis of immune-related genes or gluconeogenesis [19]; thus, a lowered or retarded local expression of IL-1β could delay the starting of the healing process. In fact, differences on the expression of cytokines were observed on those studies; in rainbow trout, amongst all cytokines evaluated, including IL-1β, only the expression of IL-6 was upregulated by day 14 post-wounding and β-glucan was found to have no effect on wound healing [12]. In contrast, in common carp, β-glucan bathing augmented the expression of cytokines key to the inflammatory reaction (IL-1β and IL-6) in the skin and IL-8 in muscle [11] by day 3 post-

wounding. β-Glucan in known to orchestrate the innate immune response [10] and to improve the expression of several cytokine genes including IL-1β and, as such, might contribute to tissue healing as observed for common carp. For silver catfish, unfortunately, the nucleotide sequences of these cytokine genes are not available and the expression of cytokine genes could not be evaluated.

Skin and muscle lesions as reported here are stressful conditions that might induce the production of cortisol. Cortisol production inhibits the expression of IL-1β [20,21] which is central to the inflammatory reaction that initiates wound healing. In contrast, β-glucan increases the expression of IL-1β [10]and related cytokines and should improve wound healing. In our study we demonstrated that at day one post-wounding cortisol levels peaked but were significantly lower in fish bathed with 0.5% β-glucan. In this sense, lower cortisol levels have lower impact on the expression of IL-1β and, as such, the inflammatory phase of wound healing might be less affected on 0.5% β-glucan treated fish than on control wounded fish.

A throughout histopathological analysis of skin and muscle regeneration is still lacking for most cultivated fish species. In our study, histopathological analyses of the wounded site were performed at day 1, 7, 14, 21 and 28 post-wounding. At day 1 post-wounding we found the wounded site already covered by a thin layer of immature epidermal cells. Contrary to mammals, re-epithelialization in fish occurs by migration of the keratinocytes at the leading edge of the wound [2,6] and, in zebrafish (*Danio rerio*) for instance, it occurs at a rate of 250 μm/h; thus, by 24 h, a 3 mm wound on skin and muscle, as reported here, should be fully closed and re-epithelialized as we indeed observed on the histopathological samples from fish bathed with 0.5% β-glucan. In *Piaractus mesopotamicus*, a tropical teleost fish, re-epithelialization of the wounded area was complete by 24 h [22] and enhanced by the addition of *Saccharomyces cerevisiae* in the food. By day seven post-wounding a complete mature epidermal cell layer was also observed. In muscle, we found discrete signs of inflammation, containing mixed inflammatory cell infiltrate that contrasts to the strong inflammatory response observed on the acute phase of healing in mammals [6]. In this sense, a strong inflammatory response might cause prolonged edema and retard the progression to the next phase of healing; in addition, proinflammatory cytokines and free radical might damage skin constituents [7]. In silver catfish bathed with 0.5% β-glucan we noticed a shorter inflammation phase and improved deposition of collagen fibers and fibroblast and, as a consequence, improved regeneration of muscle and dermis. This results builds up on our previous works in which we indicated that β-glucan improves the resistance of silver catfish to an experimental infection with *Aeromonas hydrophila*; in future work it would be interesting to investigate whether addition of β-glucan to fish diet would also improve tissue wound healing.

In conclusion, we demonstrated that wound healing occurs rapidly in silver catfish and that tissue regeneration will greatly benefit from bathing with β-glucan.

Declarations of interest

None.

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