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Original Article

Wound healing effect of chitosan in fresh water fish *Cyprinus carpio L.*

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Abstract

The shell fish (crustacean) processing industry in India generates 8.5 million tones of waste per year. These wastes have an appreciable potential for pollution and do pose a disposal problem. These wastes were converted in to chitin (value added bio polymer) and chitosan by demineralizing, deproteinizing and deacetylating with 40 - 52% NaOH for 2hr boiling at 110^{o}C. Chitin / chitosan were renounced for its commercial application in biomedical, food preservation and chemical industries. In this study chitin / chitosan was used as a dietary supplement with basal diet and tested in *Cyprinus carpio* L. Effect of chitin / chitosan on wound healing in fingerling and adult *C. carpio* were studied. α-chitosan feeding promotes epithelialization, faster wound contraction and reformation of connective tissue and angiogenesis. The present study revealed that the use of α- chitosan as wound healer of carps in intensive culture conditions.

Keywords: Crustacean; chitin; chitosan; *Cyprinus carpio*

Introduction

Wound healing is the process of repair that follows injury to the skin and other soft tissues. The capacity of a wound to heal depends in part on its depth, as well as on the overall health and nutritional status of the individual (Enoch and Harding, 2003). Dietary modifications, with and herbal supplements may improve the quality of wound healing by influencing the regenerative processes or by limiting the damaging effects of inflammation (Davis *et al*., 1989).

Glucosamine sulfate and chondroitin sulfate may both play a role in wound healing by providing the raw material needed by the body to manufacture connective tissue found in skin, tendons, ligaments and joints (Morrison and Murata,1974). One controlled trial in human found that wounds healed with greater strength when they were treated topically with a chondroitin sulfate-containing powder (Prudden *et al.,* 1969). Arginine supplementation increases protein synthesis and improves wound healing in animals (Barbul *et al*., 1983). Two controlled trials have shown increased tissue synthesis in surgical wounds in people given 17 - 25 grams of oral arginine per day (Krik *et al*., 1993; Barbul *et al*., 1990).

Chitosan was used in wound dressing matrices (Jayasree *et al.,*1995) and was shown to be a wound healing accelerator (Minami *et al.,* 1993). Chitosan's cationic nature appeared to be the main mechanism by which cells are attracted to this polymer; however, the degree of cell attachment is attributed to the percent deacetylation of the chitosan. Prasitslip *et al.* (2000) studied how degree of deacetylation affected *in vitro* cellular responses of chitosan from two different sources, shrimp and cuttle fish. They tested four chitosan substrates, two from each source, differing by about 10% in deacetylation and ranging between 76% and 90% deacetylation. Results indicated that cells more readily attached to more deacetylated chitosans from both sources. The *in vivo* chitosan tissue biocompatibility studies tended to be in agreement with the *in vitro* cell response studies. *In vivo* studies of tissue response to films of chitosan showed a marked increase in chitosan-tissue biocompatibility as chitosan deacetylation increased (Tomihata and Ikada, 1997). The present study examines the effect of chitin and chitosan supplemented diets on the proliferation of fish epithelial cells and time in healing of wounds in *Cyprinus carpio* (L.).

Material and Methods

Experimental design

Cyprinus carpio fingerlings of mean body weight of 56.9 ± 0.5 g were randomly distributed into six 250 liter tanks supplied with partially recirculating ground water at 25^{o}C. The experimental tanks were assigned to each of four dietary treatments containing 1%each of α, β-

chitin, *α, β*- chitosan. After 6 weeks of acclimation *C. carpio* were fed with basal diet and basal diet supplemented with 1%each of α, β- chitin and *α, β*- chitosan. The fish were fed with the experimental diets for a period of 4 weeks. At the end of the experimental period, fish were experimentally wounded and the responses of wound closure were observed for another 3 weeks by feeding the basal and chitin/ chitosan supplemented diets. Wound infliction consisted of a small incision measuring 1.5 ± 0.2cm in length and 0.3cm in depth made on each lateral side of the fish with a scalpel and a plastic guide. The incision penetrated the epidermis, dermis and underlying musculature. Measurement of wound closure was as per earlier descriptions (Lim *et al.,* 2004).

$$\text{Wound closure} = \frac{\text{Final wound (cm)}}{\text{Initial wound (cm)}} \times 100$$

Wound from individual fish was photographed every day, beginning on the day of wounding. Wound size was then calculated by determining the area of the wound in comparison to the standard. Wound closure was expressed as the ratio of wound area (each day after wounding). A smaller wound ratio indicated faster wound closure.

Statistical analysis

Data were reported as mean ± standard deviation. Statistical significance of the influence of diet on wound size were determined at each time using Student '*t*' test at 5% level of significance.

Result

Wound healing responses in fingerlings

Wound healing influence of chitin/ chitosan supplemented diet was studied in *C. carpio* fingerlings for a period of 35 days. The experimental fishes fed with *α*- chitosan supplemented diet showed significant reduction in the wound dimensions than all other experimental diets. On 35th day the wound was completely healed in α- chitosan supplement diet only. About 3.70% was cured in the fishes fed with basal diet after 35th day. 31.25% was healed in the fishes fed with α- chitin supplement diet and 26.67% wound closure was observed in fishes fed with *β*- chitin supplemented diet. After 35th day, 68.75% wound closure was observed in β- chitosan supplemented carps. Significant wound healing was observed in *α, β*- chitosan, *α*- chitin supplemented diets fed *C. carpio* fingerlings after 35 days (P<0.05) (Table - 1).

Table - 1: *C. carpio*: Wound closure responses of fingerlings fed with chitin / chitosan supplemented diet for a period of 35 days

Feed type	Wound size (cm)						Healing response (%)
	Initial	Day 7	Day 14	Day 21	Day 28	Day 35	
Basal diet (BD)	1.35	1.35	1.40	1.50	1.40	1.3	3.70
BD + α- chitin	1.60	1.55	1.40	1.30	1.20	1.10	31.25*
BD + β- chitin	1.50	1.45	1.40	1.30	1.20	1.10	26.67*
BD + α- chitosan	1.60	1.40	0.95	0.61	0.28	C. H	C. H *
BD + β- chitosan	1.60	1.45	1.20	0.95	0.75	0.50	68.75*

C. H - Completely healed, * indicates significance (P< 0.05) level.

Wound healing effect in adults carps

Wound healing influence of chitin/ chitosan supplemented diet was studied in adult carps for a period of 28 days. The experimental fishes fed with *α*- chitosan supplemented diet showed significant reduction in the wound dimensions than all other experimental diets. On 28th day the wound was completely healed in α- chitosan supplement diet, only about 6.670% was cured in the fishes fed with basal diet after 28th day, 43.75% was healed in the fishes fed with α- chitin supplemented diet and 40.00% wound closure was observed in fishes fed with *β*- chitin supplemented diet. After 28th day significant response in (93.33%) wound closure was observed in *β*- chitosan supplemented carps. Wound healing was significant in *α, β*- chitosan, *α*- chitin supplemented diets fed *C. carpio* after 28th days (P<0.05) (Table - 2).

Table - 2: *C. carpio*: Wound closure responses of adult fish fed with chitin / chitosan supplemented diet for a period of 28 days

Feed type	Wound size (cm)					Healing Response (%)
	Initial	Day 7	Day 14	Day 21	Day 28	
Basal diet (BD)	1.50	1.55	1.50	1.45	1.40	6.67
BD + α- chitin	1.60	1.50	1.30	1.10	0.90	43.75*
BD + β- chitin	1.50	1.40	1.30	1.10	0.90	40.00*
BD + α- chitosan	1.60	1.10	0.60	0.25	C. H	C. H *
BD + β- chitosan	1.50	1.10	0.85	0.45	0.10	93.33*

C. H – Completely healed, * indicates significance (P< 0.05) level.

Discussion

Healing of skin wound was quite a complicated process involving epidermal regeneration, fibroblast proliferation, neovascularization and synthesis. Many investigators studied the acceleration of wound healing process and shortening of healing period. Supplementation of chitin/ chitosan diet improved the wound healing efficiency of *C. carpio* and accelerated healing period (Conti *et al*., 2000). Chitosan helped the regeneration of tissue elements in skin wound and had positive effects on wound healing (Bartone and Adickes, 1988).

Supplementation of α and β- chitosan in the diet significantly improved the wound closure in the experimental carps. Similarly application of water soluble chitosan solution resulted in complete re-epithelialization, fibrosis and regrowth of hair follicles within 7 days after wounding (Cho *et al*., 1999). Ueno *et al*. (1999) reported that on the 3rd day after wounding, the chitosan treated wounds showed heavy infiltration of polymorphonuclear cells and an increase in the diffusion compared with other experimental groups in dogs. Granulation was more intense after chitosan treated dog on day 9th and 15th day of post wounding (Ueno *et al*., 1999).

It was anticipated in the experimental studies whether the duration of wound healing could be shortened. Some studies had revealed that exogenous application of growth factors decreased the healing period (Fu *et al*., 1998; Gilpin *et al*., 1994). Quilhac and Sire (1999) examined the dynamics of re-epithelialization process after wounding of cichlids fish and observed a rapid differentiation of the epidermal basal layer cells. In comparison with the epidermis, repair of dermal and muscle structure took much longer and they not reached steady state level in wound site within the period investigated. This finding was supported by Halver (1972) who found a prolonged repair period for epidermal repair. The 15th day after operation untreated control group did not heal and covered with large crust in wounded dog (Kweon *et al*.,2003). Application of water soluble chitosan ointment at wound site may not completely resulted in the healing of wound but improved the healing process and decreased the size of lesion than that of control group (Kweon *et al*., 2003).

Wound healing experiments using this fish model (*C.carpio*) showed that the application of dietary supplementation of chitin/ chitosan on to an open wound induces significant wound contraction and accelerates the wound closure and healing time. The present study revealed that the use of α- chitosan as wound healer of carps in intensive culture conditions.

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