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In vivo Studies of Chitosan Fiber Resorption

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Summary

Scanning electron microscopy and histologic analysis were used in the comparative *in vivo* study of resorption of chitosan fibers implanted into endomysium and perimysium of a rat *latissimus dorsi* muscle. It was demonstrated that the mechanism and rate of chitosan fiber resorption depend on the position of fibers in muscular tissue. After implantation of chitosan fibers into endomysium (when chitosan was in direct contact with muscle fibers), the formation of cross-sectional cracks, fragmentation of implanted fibers and its partial resorption were observed in 14 days. Complete chitosan resorption in endomysium occurred after 30 days only. Chitosan fibers implanted into perimysium preserved integrity for 7 days, and fibrous tissue was formed around implants. After 45 days of exposure, no signs of chitosan fiber destruction were registered in this case.

Riassunto

Le analisi con il microscopio a scansione sono state utilizzate assieme ad indagini istologiche per verificare il riassorbimento *in vivo* di fibre di chitosano a livello di endomisio e perimisio del muscolo del dorso del ratto. È stato dimostrato che il grado di assorbimento ed il relativo meccanismo d'azione delle fibre di chitosano dipendono dalla posizione specifica delle fibre nel tessuto muscolare.

Dopo 4 giorni dall'applicazione del chitosano nell'endomisio (quando cioè il chitosano era in diretto contatto con le fibre muscolari), sono state osservate fessurazioni e frammentazioni a livello delle fibre impiantate a cui ha fatto seguito un loro parziale assorbimento.

Le fibre di chitosano impiantate nel perimisio hanno conservato una loro integrità per 7 giorni con formazione di tessuto fibroso che le ricopriva.

Dopo 45 giorni il chitosano veniva completamente riassorbito.

INTRODUCTION

The development of materials capable of substituting for lost organ or its parts is the main goal of tissue engineering and transplantology [1-3]. The chitosan (polysaccharide obtained from natural polymer chitin) is of great interest for development of scaffold materials. It is known that chitosan is biocompatible and decomposes in biologically active media [4,5].

A literature analysis demonstrates that the resorption rate of chitosan materials depends on its deacetylation degree [6,7], molecular mass [8], porosity [9], the presence of nanoparticles or other polymers in film and bulk samples [10]. The majority of data on chitosan resorption were obtained during *in vitro* experiments, in lysozyme-containing media or *in vivo* after subcutaneous implantation of the studied chitosan material.

As a rule, the used objects were small films or plates. There are virtually no publications concerning *in vivo* studies of the mechanism of chitosan fiber resorption, although these fibers may be used as one-dimensional matrices in tissue engineering (e.g., prototypes for nervous tissue, muscles and ligaments). Therefore, information about kinetics of *in vivo* resorption of chitosan fibers is of special interest.

The aim of the present work is comparison between the mechanism of resorption of chitosan fibers implanted into endomysium (when maximum contact between chitosan and muscular tissue is observed) and into perimysium (in close proximity to muscular tissue, but without direct contact to a muscle).

MATERIALS AND METHODS

Chitosan (Fluka Chemie, BioChemika line) with a molecular mass of 225 kDa, deacetylation degree of 80% and ash content of 0.5% was used.

The fibers prepared from this chitosan by the method described elsewhere [11] were used in the work too. Their tensile strength, tensile modulus, deformation at break of the chitosan fibers were 220 ± 10 MPa, 900 ± 100 MPa and 8 ± 1 % correspondingly.

The experiments were performed using 20 female white Wistar rats in accordance with good laboratory practice, the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and the World Medical Association's Declaration of Helsinki (1996) concerning humane treatment of laboratory animals.

The weight of test animals was 180-200 g, the age was 6 months.

In the studies of the *in vivo* resorption, a bundle of chitosan fibers 20 mm in length containing 10 monofilaments with a diameter of 80 μm was sterilized in 70 wt.% ethanol for 1 h.

The operated animals received inhalational anesthesia (3% isoflurane). The fibers were placed into a *latissimus dorsi* muscle, and then the wound was stitched up layer wise with atraumatic suture needles using a Prolen 4-0 thread.

Chitosan fibers were implanted into the latissimus dorsi muscle in the endomysium between individual muscle fibers and perimysium between bundles of muscle fibers.

The position of the fibers during implantation is shown in Figures 1 a, b. Implantation of chitosan fibers into endomysium was performed using 10 animals. Muscle fibers were spaced about 2 mm apart with tweezers, and no more than two chitosan fibers were introduced into the resulting cavity (20-25 mm in length), see Figure 1 a.

In the experiments with other group of animals (10 individuals), a bundle of chitosan fibers was applied over a bundle of rat *latissimus dorsi* muscle, i.e., into perimysium (Fig. 1b).

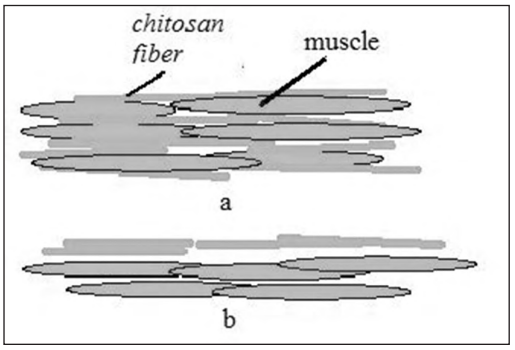

Fig. 1 *Implantation of chitosan fibers into endomysium (a) and perimysium (b).*

After suturing, animals were kept in individual cages and had free access to water and the standard fodder. All animals were active after surgery; no negative influence of implantation was revealed. The implanted fibers were extracted from animals at 7 days intervals.

Histologic analysis

Morphological studies were performed in 1 and 6 weeks after the surgery using muscle tissue with chitosan fibers and adjacent connective tissue. The samples were fixed in 10% neutral phosphate-buffered formalin (pH=7.4) for 24 hrs, dehydrated in a series of ethanol solutions of increasing concentrations and embedded into paraffin blocks according to the standard histological technique. Transverse sections of muscle and chitosan fibers 5 μm thick were prepared. The sections were colored with hematoxylin and eosin (Bio-Optica, Italy) and according to the Mallory method (Bio-Optica, Italy).

Microscopy analysis was performed with the use of a Leica DM750 light microscope (Germany) at x100 and x400 magnifications. Photographic survey was performed with a Leica DM750 microscope and an ICC50 camera (Leica, Germany).

Fiber samples before and after implantations were fixed on object tables and spattered by gold. The samples were studied using a Supra 55VP scanning electron microscope (Carl Zeiss, Germany) in the secondary electron mode.

RESULT

Resorption of chitosan fibers in endomysium

Figure 2 presents a micrograph of chitosan fiber surface (Fig. 2 a) and its cross-section made in liquid nitrogen (Fig. 2 b). It can be seen that the surface of the fiber is smooth and contains no defects. The cross-section of a fiber is also dense; no large pores or cracks were detected. After implantation of these fibers in a rat muscle (endomysium), in 7-14 days cross-sectional cracks are formed on the surface. The width of these cracks increases with exposure time. Figures 2 c, d gives micrographs of fibers exposed in endomysium for 14 days. It may be presumed that these cross-sectional cracks result from mechanical dynamic load which affected chitosan fibers placed in direct contact with muscles. Throughout the experiment, all animals were active; their muscles were repeatedly stretched and constricted in different directions, thus leading to destruction of chitosan fibers contacting with muscle tissue.

The studies of implantation area have demonstrated that after 14 days the fragments of fibers 3-5 mm long are formed, and these fragments are partially resorbed. It should be noted that resorption process virtually does not change fiber diameter and their biodegradation starts from the ends.

This fact is evidenced by the results of the studies of fiber cross-section after exposure in animal organism (endomysium). Figures 2 e and 2 f demonstrate the presence of a large amount of pores in the interfibrillar space (which were not observed in the initial fibers). It can be assumed that the active biological medium containing

enzymes and macrophages penetrates into interfibrillar spaces of fragmentary fibers at the expense of capillary forces and leads to intensive chitosan degradation.

Observations of the implantation zone demonstrated that after 21 days only low amount of fiber fragments is present.

The main portion of chitosan fiber degrades, and degradation products are removed from the implantation zone during metabolic processes. In 30 days, neither fibers nor their fragments were observed in the implantation zone; all chitosan fibers were completely resorbed.

The resorption process is shown in Figure 3.

The important thing to note is that throughout the experiment (30 days) no connective tissue was formed around chitosan fibers or their fragments, and thus, encapsulation did not occur.

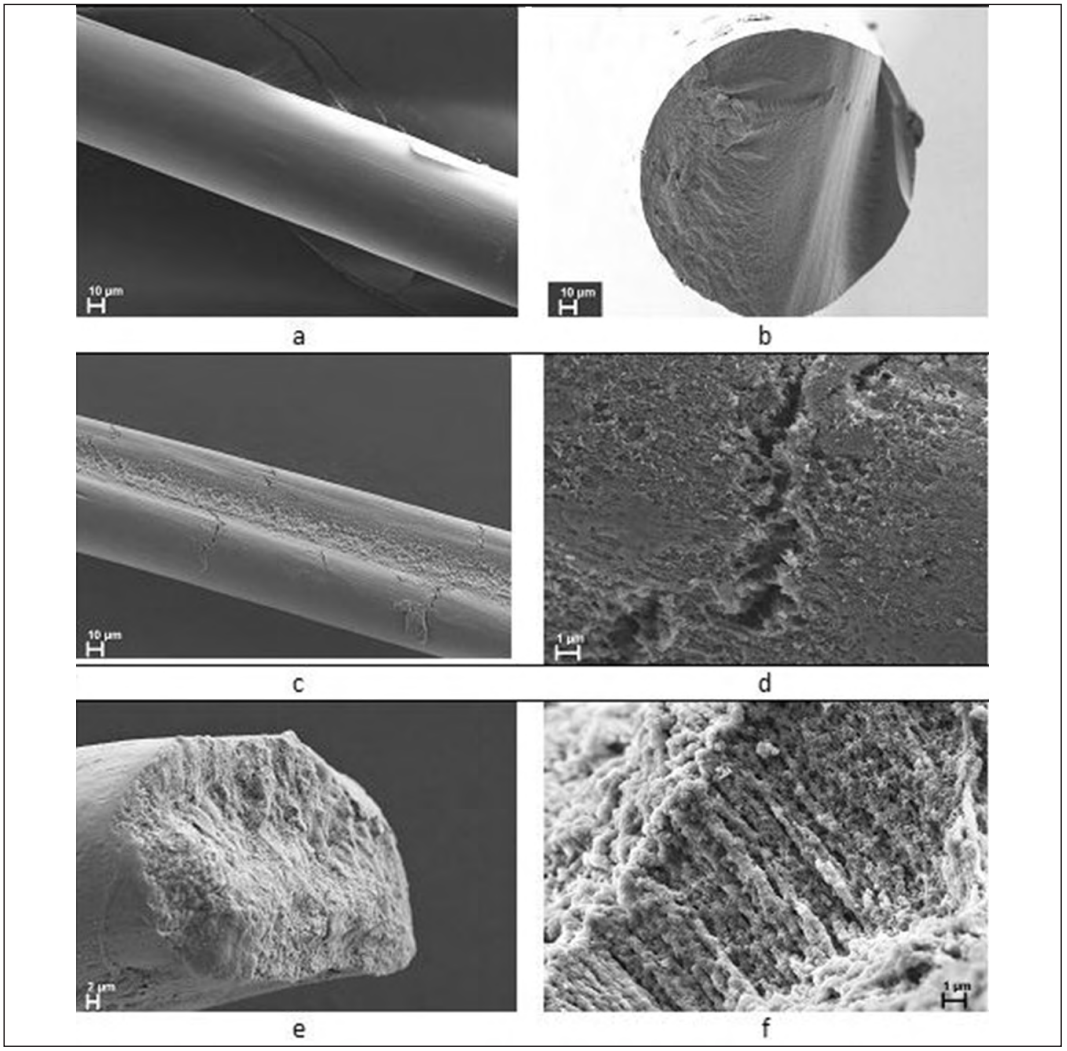

Fig. 2 *Micrographs of lateral surface (a) and cross-section (b) of the initial chitosan fibers and the fibers removed after exposure to rat endomysium for 14 (c, d) and 21 (e, f) days.*

Resorption of chitosan fibers in perimysium

Another picture was observed after implantation of the fibers into perimysium. The studies of the implantation zone showed that after 7 days the fibers remained intact. After prolonged exposure (6 weeks) no qualitative changes in the implanted fibers were observed, but fibrous tissue was formed around them, as evidenced by histological analysis of the chitosan fibers exposed to rat organism (Fig. 4).

It is clearly seen that fibrous capsules were formed around every chitosan fiber. Cell infiltrate of fibrous tissue includes individual multinucleated cells of foreign bodies and insignificant amount of macrophages, monocytes and lymphocytes, this indicating aseptic chronic inflammation.

Considerable differences in behavior of chitosan fibers implanted into muscle endomysium and perimysium may be explained by mechanical stresses appearing in rat latissimus dorsi muscles due to animal activity and affecting the fibers implanted into endomysium.

These multidirectional dynamic loads have an impact on the implanted chitosan fibers and facilitate their degradation.

The important factor influencing accelerated fiber resorption is an intensive metabolism in muscle tissue. Besides, it is expected that continuous shear stresses affecting the implanted fibers, their mutual friction and friction between chitosan and muscle bundles (due to muscle stretching and contracting) prevent the formation of connective tissue around chitosan fibers.

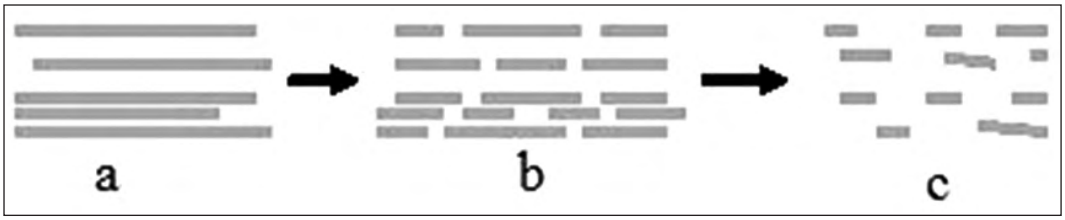

Fig. 3 *Resorption of chitosan fibers in rat latissimus dorsi muscle endomysium: a — the initial fibers; b, c — the fibers exposed to endomysium for 14 (b) and 21 (c) days.*

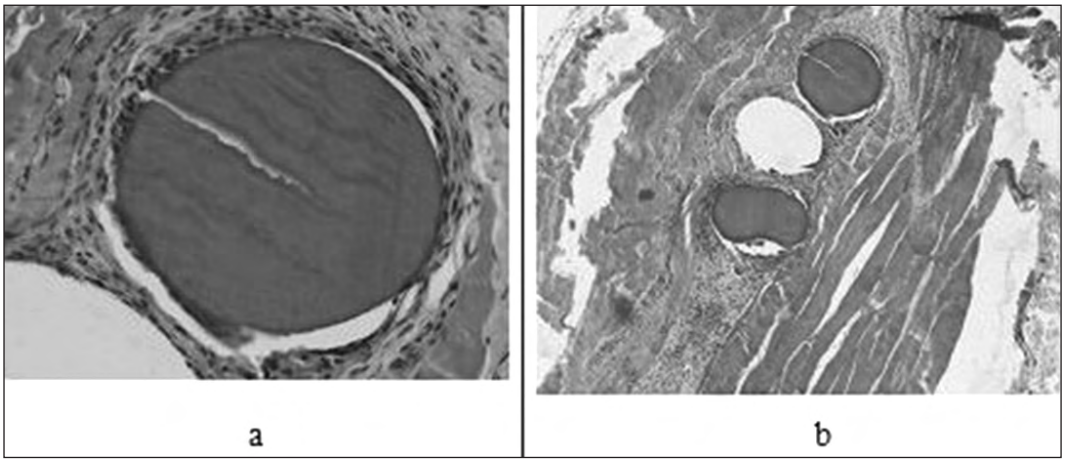

Fig. 4 *Cross-section of the chitosan fibers after 6 months implantation in perimysium; colored with hematoxylin and eosin; magnification a - x400 and b - x100. Around fibers revealed fibrous capsule.*

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Chitosan fibers implanted into perimysium were subjected to smaller mechanical loading, and metabolism in these areas is rather slow. Therefore, the implanted fibers were encapsulated in a fibrous capsule.

The resorption rate in this case is significantly lower.

CONCLUSION

The studies of resorption of chitosan fibers implanted into rat *latissimus dorsi* muscle demonstrated that the position of these fibers in muscle tissue has a strong influence on the resorption mechanism.

The fibers located in endomysium (in direct contact with muscle tissue) were subjected to multidirectional loading. This loading led to the formation of cross-sectional cracks and fragmentation which took place 14 days after the surgery.

Diffusion of active biological medium (containing enzymes and macrophages) into interfibrillar spaces of fiber fragments facilitated their complete resorption in 30 days.

Chitosan fibers implanted into perimysium (where mechanical stresses and metabolism rate are lower than those in muscle interfibrillar space) were encapsulated in 7 days after the implantation.

The resorption process was significantly slower as compared to the previous case.

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