A study of the relationship between mass and physical strength of keratin bars *in vivo*

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A study was undertaken of the changes in the mass and physical properties of keratin bars implanted subcutaneously in adult rats. A very gradual decrease occurred *in vivo* in the dry weight of the bars over the period of the study (up to 18 weeks). The elastic modulus of the bars decreased abruptly when present *in vivo* between 3 and 6 weeks. At the same time there was an increase in the number of cavitations and fissures at the surface of the bars, and an increase in a central internal region of the bars where there was a disorganisation in structure of the polymer. A biocompatible material showing such changes *in vivo* is likely to be suitable for a variety of medical and surgical applications in which it provides a framework for cell invasion.

1. Introduction

There are a number of porous biocompatible materials that are implanted in tissues during surgery for the purposes of repair, reconstruction, and augmentation. They can be divided into the two main categories of non-biodegradable materials, which include metals [1], and biodegradable materials, such as collagen [2, 3], polylactic acid, polyglycolic acid and their copolymers [4]. The biodegradable materials that have been studied most are polymers and include proteins, polysaccharides, polyaliphatic acids, and polyesters. They have extensive uses in surgery because of their resorbability, leading to their replacement by normal tissue over a period of time (e.g., collagen and oxidised cellulose [5]; polyurethane [6]). Over the last decade there have been many studies made of the extent of resorbability of such materials, which primarily is indicated by a loss of mass [7, 8]. With the resorption of these materials, there is the likelihood of an accompanying decrease in physical strength [7].

We chose to carry out a study to examine the relationship between mass and physical strength using a polymeric material that was biodegradable, of adequate physical strength, and could be obtained in high purity. Collagen is not suited because of its inadequate strength (although having high tensile strength, it has low compressive strength [9, 10]). Polylactic acid or poly(lactide-co-glycolide) is also unsuitable since, although each has high physical strength, they have very low biodegradability and there is the possibility of tissue toxicity due to acidic breakdown products [1, 11]. For these reasons we chose to use reconstituted keratin in the form of bars manufactured by the Wool Research Organisation of New Zealand. These bars have adequate physical strength (including tensile and compressive strength) and biodegradability. The collection of such data would be extremely important in devising different medical and surgical applications for this material. For example *in vivo*, if the mass and physical strength of a such material decrease equally or if the physical strength is affected to a greater extent than the mass, it would suggest it is suitable as a resorbable implant material for non-load bearing applications. In these situations the main aim is for the material to provide a scaffold for cell invasion and the laying down of new tissue [12]. However, if the physical strength and the mass remain relatively unaffected or if the physical strength is less affected than the mass, then the material would be suitable in applications to provide mechanical support to tissues over a specific timeframe [8, 13].

2. Materials and methods

2.1. Material

Rectangular bars of reconstituted keratin polymer 12 × 4 × 3 mm (using keratin extracted from sheep wool) were supplied by the Wool Research Organisation of New Zealand, Springs Road and Gerald Street, Lincoln, Canterbury, New Zealand. These bars had been washed in water and dried in air at 20 C. The dry weights of the bars were measured. Also the external dimensions of the bars were determined using electronic calipers. The bars were individually packed in sealed plastic envelopes, numbered, and sterilized by gamma radiation (dosage 2.8 Mrads and certified to be sterile, Schering-Plough, 33 Whakatiki Street, Wellington, New Zealand).

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2.2. Experimental procedure

Eighteen adult female Wistar rats (body weight approximately 180 kg) and eight adult male Wistar rats (body weight approximately 200 g) were supplied by the Department of Laboratory Animal Sciences, University of Otago. They were allowed free access to food and water. Approval for this study was obtained from the Animal Ethics Committee, University of Otago.

Anaesthesia was induced in each animal with 4% halothane and maintained with approximately 2% halothane using the nose cone of a small-animal anaesthetic machine. Sterile clear plastic drapes were placed over the animal enabling the respirations to be monitored during surgery. The skin on the dorsal aspect of the abdomen was shaven, disinfected, and two short median skin incisions, each 6 mm length and separated by 2.5 cm, were made. A subcutaneous tunnel 2.5 cm length was made on each side of the skin incisions and a keratin bar inserted and positioned at the end of each tunnel. Four bars were randomly allocated to these sites in each animal. The two skin incisions were closed with sterile metal wound clips, and the animals removed from the anaesthetic machine and allowed to regain consciousness. The operations on each rat were completed within 5–10 min, and all except one recovered from the halothane anaesthesia very quickly. The rats became mobile within about 15 min. One female rat did not survive the anaesthesia. The animals were monitored daily for a period of two weeks, including measurement of body weight and observations of, for example, physical activity, the nature of the coat, appearance of the surgical sites and possible erythema at the sites. The rats were euthanized at selected time points using a carbon dioxide chamber. The keratin bars were removed from each of the animals, cleaned of any adherent tissues, washed over 3–5 days in several changes of distilled water, and dried in the air at 20 C. Two of the bars were randomly allocated for measuring weight, while the other two bars were used to determine physical strength and morphology.

2.3. Measurement of changes in weight and physical properties of bars

Extent of degradation: The weight of the each of the dried bars (i.e., residual mass) was calculated as a percentage of the initial dry weight. The extent of degradation of the bars occurring *in vivo* was obtained by subtracting this value from 100%. The average of two values was obtained for the residual mass and also for the extent of degradation of the bars in each rat.

Modulus of elasticity: All the measurements of the modulus of elasticity were performed under ambient conditions using an Instron machine fitted with a 3-point bending apparatus in which the knife-edge supports for the bar were 8 mm apart [14]. The bars were placed lengthwise across the supports, with the surface pointing upwards being randomly selected to average out any differences in strength due to variations in manufacturing the bars. The force applied and the deflection produced at the centre point between the two supports were recorded using a Mac Lab 4 (Apple Macintosh). Prior to testing, the dimensions of the bars were measured using electronic calipers. For each rat, the average of two values was recorded for the modulus of elasticity. The modulus of elasticity was also measured for two bars that had not been inserted into rats but had been washed with distilled water and dried in an identical way to those removed from the animals. The average of these measurements was taken to be the value at zero time ($t = 0$).

Surface and internal morphology: Using the parts of the bars recovered following fracturing in the physical strength testing experiments, the surface and the internal features of the bars were examined under a stereo microscope.

3. Results

3.1. Surgical procedure

Monitoring of the animals in the immediate postoperative period did not show any adverse response to the insertion of the keratin bars. The rats showed normal activity and alertness, and normal increase in body weight. There was little, if any change in the appearance of the coat and only a very mild erythema at the surgical sites was observed in a few animals. The skin incisions healed quickly and the metal wound clips were removed after 14 days. The times at which the rats were euthanized are given in Table I.

3.2. Weights and physical properties of keratin bars

Extent of biodegradation: The dry weights of the bars, expressed as a percentage of the initial dry weight, were not markedly different for the female rats compared to the male rats at the chosen time points. For this reason, the data for bars removed from the female rats and the male rats have been combined, and is presented in Table II. Small significant decreases of 7, 11, 15 and

TABLE I Study design regarding numbers of animals and time of euthanasia

Time	Number of female rats	Number of male rats
1 week	3	0
2 weeks	3	0
3 weeks	3	3
6 weeks	3	3
12 weeks	3	2
18 weeks	2	0

TABLE II Weights, expressed as a % of initial weight, and extent of degradation of dried keratin bars removed from rats

Time	Weight of keratin bar	Extent of degradation
1 week	97.0 ± 0.9^{a}	3.0 ± 0.9^{p}
2 weeks	92.6 ± 1.1^{b}	7.4 ± 1.1^{q}
3 weeks	93.2 ± 0.9^{b}	6.8 ± 0.9^{q}
6 weeks	89.0 ± 0.6^{c}	11.0 ± 0.6^{r}
12 weeks	84.8 ± 1.2^{d}	15.2 ± 1.2^{s}
18 weeks	77.7 ± 2.1^{e}	22.3 ± 2.1^{t}

Values are Means ± SE.

By one-way analysis of variance and Duncan's multiple range test, the means with the same superscript fell into the same subset and were significantly different from those in other subsets ($P < 0.05$).

TABLE III Modulus of elasticity of dried keratin bars at zero time and removed from rats

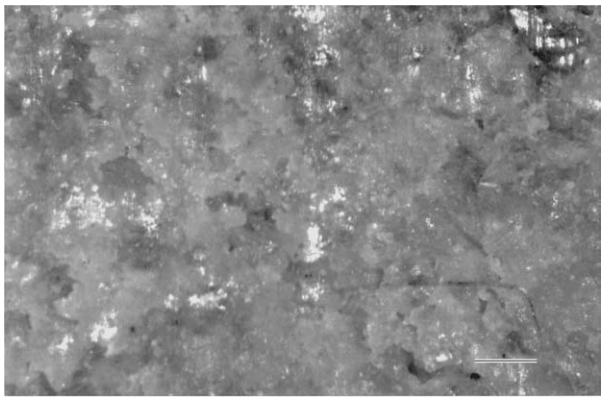
Time	Elastic modulus (MPa)
zero	717 ± 147^{a}
1 week	$592 \pm 22^{a\,b}$
2 weeks	$629 \pm 18^{a\,b}$
3 weeks	553 ± 56^{b}
6 weeks	142 ± 8^{c}
12 weeks	118 ± 11^{c}
18 weeks	47 ± 8^{c}

Values are Means ± SE.
The value at zero time ($t = 0$) is the average for two bars not inserted into rats but washed and dried in an identical way to those bars removed from the animals. By one-way analysis of variance and Duncan's multiple range test, the means with the same superscript fell into the same subset and were significantly different from those in other subsets ($P < 0.05$).

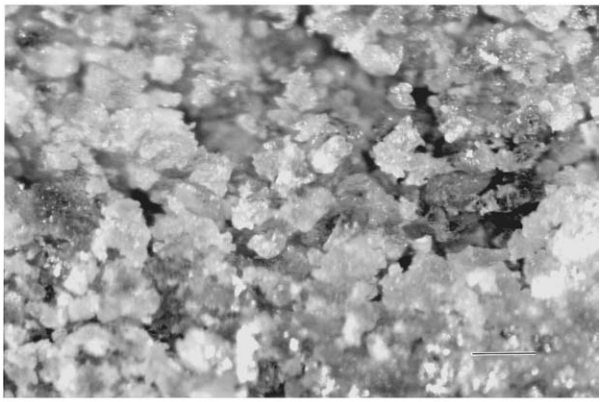
22% occurred in the mean dry weights of the bars at 3, 6, 12 and 18 weeks, respectively.

Modulus of elasticity: The moduli for the keratin bars were not markedly different for the female rats compared to the male rats at the chosen time points. The data for bars removed from the female rats and the male rats have been combined, and is given in Table III. The mean values over the first three weeks were not significantly different from one another. However, at 6 weeks there was a marked decrease in the modulus, and the value at this time was significantly different from that at 3 weeks. The means at 6, 12 and 18 weeks were not significantly different from each other.

Surface and internal morphology: For all the bars that were removed from the animals, each of the two larger surfaces (12 × 4 mm) showed cavitations and fissures. The two smaller surfaces (12 × 3 mm) showed small pits, as well as fissures that were less extensively formed than those found on the two larger surfaces. The numbers and extent of the cavitations, fissures, and pits appeared to increase with the time that the bars had been present *in vivo* (Fig. 1(a)). Examination of the bars broken across the middle from the testing of physical strength experiments, showed an inner core where there appeared to be a deficiency in the polymer matrix. This again was more pronounced in the bars removed from the animals after a longer time (Fig. 1(b)). In addition, four bars that had not been inserted into rats or washed and dried showed very similar features to those removed from the animals at the earlier times of 1 to 3 weeks.



(a)



(b)

Figure 1 (a) Surface features of keratin bars removed from rats. The surface appearance was similar for the bars removed after 6 and 12 weeks, with there being a large number of cavitations and fissures. A smaller number of cavitations and fissures at the surface were found for bars removed after 3 weeks, whose appearance was very similar to bars not inserted into animals. (b) Internal features of keratin bars removed from rats and fractured in the strength testing experiments. Examination of the fractured surfaces shows that after 6 and 12 weeks, there is a central region where the structure of the polymeric matrix is less well organised than in the outer region of the bar. This change in morphology is more advanced compared to bars removed at 3 weeks. In (a) and (b) the calibration line represents 0.1 cm.

4. Discussion

A number of different biocompatible materials are being used in the repair, reconstruction, and augmentation of hard and soft tissues in surgery. These include collagen, calcium phosphates (e.g., hydroxyapatite, tricalcium phosphate), alginates, and polylactic acid, polyglycolic acid and their copolymers. At present there is a major interest in developing resorbable materials for this purpose, for example collagen, hydroxyapatite, oxidised cellulose, alginates [15, 16]. The choice of surgical applications for these materials is determined to a large degree by the changes in their mass and physical properties following implantation in tissues. Reconstituted keratin polymer has been selected as the material model for this study because of its relatively high physical strength compared to other currently available materials such as collagen, and for its ease of handling compared to hydroxyapatite owing to its higher tensile properties [17].

In the present study it was shown that keratin bars implanted subcutaneously in rats underwent a slow degradation over a period of 18 weeks, with a 22% loss in dry weight occurring at this time. Hence *in vivo*, the reconstituted keratin bars were lysed only to a very small extent by the action of proteinases in tissue fluids and/or secreted by various cells (e.g., polymorphonuclear leucocytes, macrophages). This small amount of proteolysis is similar to that reported for polymers formed by the cross-linking of other materials [18]. However, while only small changes occurred in the mass of the keratin bars *in vivo*, there was a marked change in physical strength as indicated by the measurement of the elastic modulus. This parameter decreased rapidly at 6 weeks. The keratin bars removed from the rats had been extensively washed in water to remove tissue fluid and cellular material. The bars were then dried under

ambient conditions to constant weight, with a residual moisture content of 10% by weight (in agreement with [19]), and the same procedure was used for all the keratin bars examined. The strengths were measured of dried bars on account of it being extremely difficult to measure the modulus of elasticity of bars that had been hydrated in aqueous medium, due to softening and becoming rubber-like. Some decrease in physical strength of the bars may have resulted from washing in water prior to drying, but this was not determined owing to being supplied with a limited number of bars. It was felt that these measurements would provide information relating to possible structural changes in the keratin bars *in vivo*. In support of this, pronounced alterations were evident in the surface and internal features of the bars removed from the rats at the later times of 6 to 18 weeks. An increase was observed in the number and extent of the crevices and fissures on the surfaces of the bars, and there was an inner core of the polymer for which the three-dimensional structure was less well maintained. The surface features of the keratin bars removed at the earlier times of 1 to 3 weeks were very similar to those of bars that had not been inserted into rats or washed and dried i.e., they were present in the bars as supplied. While it is possible that subsequent washing and drying of the keratin bars removed from rats might cause some crevices and fissures to open up on the surface, the finding that these features were much more prominent in bars removed at the later times would indicate that these changes have occurred mainly while the bars are *in vivo*. It is therefore considered that the decrease in physical strength of the dried bars at 6 to 18 weeks is a reflection of changes in internal structure and form of the bars occurring *in vivo*.

Two possible explanations are suggested for these changes in morphology. Firstly, while only a limited extent of degradation of the bars occurred *in vivo*, it seems likely that proteolysis would occur preferentially at the surfaces of the bars. This may cause a lengthening and deepening of the crevices and fissures at the surface. Secondly, the reconstituted keratin polymer is hydrophilic on account of the large proportion of negatively charged amino acids present [17]. With absorption of water, the keratin bars would swell. Polymer swelling in a aqueous environment has been shown to occur with other materials e.g., poly(DL-lactide-coglycolide) [20]. To what extent hydration of the keratin bars in an aqueous medium affects the size of crevices and fissures at the surface has not been determined owing to a limited supply of bars.

From this study there is no direct relationship between the *in vivo* degradation (measured as the decrease in mass) and the physical strength of the dried keratin bars. That the physical properties of the keratin bars are more abruptly affected than the mass, and which is consistent with a change in internal structure and form of the keratin polymer, would indicate that this form of reconstituted keratin is more suited as a resorbable implant material to provide a scaffold in non-load bearing applications.

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