The Effect of a Keratin Hydrogel Coating on Osseointegration: An Histological Comparison of Coated and Non-coated Dental Titanium Implants in an Ovine Model

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Abstract Bioactive substances may be used to enhance the rate and quantity of bone healing during osseointegration of titanium dental implants. A pilot observational study was undertaken to assess a novel keratin hydrogel in six adult sheep utilising the femoral condyles as the surgical site to assess osseointegration. Implants and osteotomy sites were coated with the keratin gel prior to implant placement (test implants) whereas the opposite knee received unmodified control implants in each animal. Fifty 3.5 mm × 7 mm Neoss dental implants were surgically implanted with a range of 3–5 Neoss dental implants placed per surgical site in each knee and allowed to heal for 5 days or 2, 4, 8, 12 and 16 weeks prior to the sheep being sacrificed. Of the 50 placed implants, 24 were used for this study and analysed via resin-embedded, undemineralised sections from test and control implants to assess the range of healing around the unloaded dental implants. These dental implants were analysed using histomorphometric methods for the best 3 consecutive threads on each side and the percentage of bone to implant contact (%BIC) was used to determine the degree of osseointegration between test and control dental implants at each time point. All implants appeared osseointegrated at the time of sacrifice. One each of the pairs of control implants at 2, 4 and 12 weeks demonstrated minimal integration histologically, with %BIC <10 %. No test implants had %BIC <35 % at any time point. Mean %BIC for test implants was higher than controls at all time points except 5 days and 2 weeks. The range from 2 to 16 weeks healing

was 39.7 % [SD 25.5 %] to 85.4 % [14.2 %] for test implants and 35.6 % [43.4 %] to 46.6 % [23.1 %] for controls. %BIC appeared to increase earlier in the test implants (from 4 weeks onwards) compared to controls. After 16 weeks, %BIC was almost twice as great in test implants as controls. This pilot observational study suggests that keratin hydrogel may promote earlier osseointegration around titanium dental implants. Further cross-sectional studies with larger sample sizes are warranted. The most marked difference between test and control implants was seen after 4 weeks. It is recommended that future studies in this model focus on healing after 4 weeks.

Keywords Dental implants · Bioactive substances · Osseointegration · Ovine model

Introduction

There has been extensive research into the physical, chemical and biological characteristics of the dental implant surface in an attempt to enhance the osseointegration process and allow earlier loading as well as to obtain faster functional use of dental implants.

Changes to the implant surface including biological mediators have been trialled and growth factors (GF) as well as bone morphogenic proteins (BMP) are current areas of interest to promote bone growth on the dental implant surface. Although both BMPs and GFs hold theoretical potential for earlier osseointegration, their method of sustained delivery continues to be challenging [1] although some experiments show the potential of biological enhancement [2].

More recently, keratin has shown promise as biomaterial filler which has been found to promote both cell induction and nerve regeneration. Keratin is readily found in hair,

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wool, nails and feathers and has a high proportion of cysteine amino acids with their high-strength disulphide bonds giving keratin the dual properties of both strength and flexibility. The hair follicle is thought to be remarkably proliferative, controlling over 30 growth factors, cytokines and signalling processes. These processes have been found to be important for regeneration of most body tissues following injury [3], including nerves [4, 5]. Sierpinski et al. [6] postulated that growth factors are retained in the keratin hydrogel providing favourable mitogenic, chemotactic and gene regulation characteristics. Therefore, it was reasonable to hypothesise that keratin has the potential to be osteoinductive when applied to bone due to the combinations of growth factors, cytokines and cellular signalling. A hydrogel formulation of keratin has been produced (Keratec) and its biocompatibility has been noted in previous studies [7, 8].

Materials and Methods

Keratin Composition

The keratin hydrogel was obtained from Keratec Ltd (Keratec Ltd. Keratec Building, 1375 Springs Road, Lincoln, Canterbury, New Zealand). The keratin gel is derived from sheep's wool and comprises "S-sulfonated keratin and hydroxyapatite in a weight ratio of S-sulfonated keratin to hydroxyapatite ranging from 1:0.1 to 1:2" (www.freepatentsonline.com/7297342.html) [9]. The patent information describes it as "biocompatible, biointegratable, and biodegradable and the primary application of the material is in orthopaedic surgery for replacement and augmentation of bone, and fixation and immobilization of bone fractures and bone segments". Prior to surgical implantation, the hydrogel was sterilised by gamma radiation and then maintained at 4 °C refrigeration until use, as advised by the manufacturer.

Dental Implants

The implants used in this study were 7 mm × 3.5 mm Neoss titanium implants (Neoss Pty. Ltd. PO Box 404. New Farm. QLD 4005 Australia). According to the manufacturer, the implant surface was subjected to a multistage blasting preparation and cleaning process, resulting in a coarse surface area over the threaded section of the implant whilst a more fine level of surface roughness was superimposed over the entire implant. This is a process called "bimodal" surface. All drills and drivers were used as per normal surgical protocols and following the manufacturers recommendations.

Ovine Model and Surgery

Ethical approval was obtained from the University of Otago Animal Ethics Committee for this study (AEC 58/06). Six adult New Zealand mixed breed sheep were used. Implants were placed under general anaesthetic into the femoral condyles according to protocol established by Duncan et al. [10]. Initially, five 7 mm × 3.5 mm Neoss titanium implants coated with the protein gel and five identical Neoss implant controls were placed in the right and left femoral condylar heads respectively with the hydrogel being injected also into the prepared test sites. The right knee was used as the test (keratin) site in all animals except the sheep used for the 12th week. In this case, the keratin was applied on the left knee.

The initial protocol was to assess healing at 2, 4, 8, 12 and 16 week intervals using five implants placed in each knee (10 implants used per animal). This had to be modified as one of the sheep was unable to weight-bear following the procedure. This was due to unsatisfactory wound closure. This animal was killed at 5 days post-operatively for humane reasons and the protocol was modified accordingly. Another sheep was utilised and three implants were used per knee (6 per animal) for the additional sheep. Therefore, the final protocol involved 50 implants placed into six sheep with healing periods of 5 days, 2, 4, 8, 12 and 16 weeks.

The animals were euthanized by the veterinarian staff via an overdose of thiopentone and prepared for unmineralised resin-embedded histology as previously described by Duncan et al. [10]. Sections were viewed using an Olympus Vanox-T microscope at 20× magnification (Olympus Australia Pty Ltd, Australia) and digital images captured using a Diagnostic Instruments SPOT RT Colour camera (SciTech Pty Ltd, Australia). Two to four sections per implant were analysed.

Evaluation

The stained sections were recorded as JPEGs (Spot Advanced Version 3.5.9 for MacOS, Mac OS 9.2, Olympus AH-2). At that magnification, each photomicrograph approximated one thread. Per cent bone-to-implant contact (%BIC) was calculated using Image J Version 1.38 (NIH, Bethesda, MD, USA). The perimeter of the implant thread was measured first followed by the length of bone surfaces (osteoid in the earlier implants) clearly in contact with the implant surface.

Results

Of the 50 implants placed, 24 were used for this study and prepared histologically as described above. The remaining

26 dental implants were retained for future research purposes. Of the 24 dental implants used for the present study, 74 histological sections were stained and 629 photomicrographs were assessed by histomorphometric analysis to determine %BIC at 20 $\times$ magnification. The more central histological slices obtained from the implant had better resolution of %BIC but some of the more lateral slices still yielded useful information.

Descriptive Histology

No overt signs of inflammation, infection or bone resorption were evident on any of the photomicrographs. The keratin product was visible under high power in the 5-day and 2 weeks samples (Fig. 1a, b) but much less obvious at the 4-week interval. Osteoid was evident (Fig. 2a, b) in the earlier implants (5 days and 2 weeks) but was largely replaced by bone at the 8-week time point (Fig. 3). Differences readily shown in the photomicrographs between the test and control implants at 8 weeks show a greater concentration of bone around the implant threads of the test implants (Fig. 4a, b).

Histomorphometric Analysis

The %BIC around results from the 3 best consecutive threads per side are shown in Table 1 and Fig. 5. As only one animal per time point was used, no statistical comparisons were performed and only descriptive statistics are presented here.

In both the control and test implants, there was initially a high %BIC 5 days following surgery which decreased in the following weeks and then slowly recovered (Figs. 5, 6). This rebound and formation of new bone occurred at an earlier time point in the test implants as can be seen in Fig. 6. From 2 to 16 weeks, test implant %BIC increased by 215 %, compared to a 130 % increase for the control implants over the same time period. However, the drop in %BIC after surgery continued for longer and reached much lower levels around the control implants, reaching 14.5 % [SD 16.25] after 4 weeks. From this low point at 4 weeks, to the final time-point at 16 weeks, the %BIC around control implants increased by 322 %. Over the same period the rate of increase around test implants appeared to slow, with % BIC increasing by 133 % from 4 to 16 weeks. Unexpectedly, three of the control implants demonstrated minimal osseointegration (<10 % BIC) at 2, 4 and 12 weeks (Table 1).

Discussion

From this observational study, there were differences in %BIC values between the test (keratin) and control

Fig. 1 Five-day test implant photomicrograph demonstrating keratin hydrogel in contact with 2nd superior thread. **a** $\times 2$ magnification. **b** $\times 20$ magnification of box in Fig. 1a

implants at most time intervals. This trend was more evident from 4 weeks onwards and indicated a higher %BIC on the test implants as compared to the controls at all subsequent intervals (Table 1; Figs. 5, 6). There appeared to be little difference in the %BIC in the early intervals (5 days and 2 weeks) between the control and test implants. As expected, there was quite a large standard deviation for all implants in the study due to the very small sample size. Regardless, the data suggests that at 4 weeks post-insertion there was a clear difference in the %BIC between the test and control implants and that the keratin hydrogel altered and likely enhanced bony healing.

As expected, both the control and test implants showed an initially higher %BIC closer to the time of surgery before decreasing in the weeks following and then slowly recovering as primary stability yielded to secondary stability. The results of this study imply that by the 4-week interval the test implants are already well under way to forming new bone around the implant surface while the %BIC was less in the controls (Figs. 5, 6). Therefore, it appeared that the reduction in primary stability was either delayed or the deposition of new bone occurred more quickly in the keratin coated implants. Either way, the test

Fig. 2 Two-week test implant photomicrograph demonstrating osteoid and osteoblast front on the 3rd superior thread. **a** $\times 2$ magnification, **b** $\times 20$ magnification of box in Fig 4a

Fig. 3 Eight-week test implant photomicrograph demonstrating %BIC of 2nd superior thread. $\times 20$ magnification

Fig. 4 Eight-week test and control implant photomicrographs demonstrating difference in bone to implant contact at $\times 2$ magnification. **a** Test implant. **b** Control implant

implants did not lose stability to the same degree as the non-keratin coated controls.

The ovine model was used for a number of reasons. This is an animal model that is gaining popularity for dental implant research mainly due to cost, ease of animal handling and a more favourable public perception with respect to other animal models (e.g. dogs). The chief Veterinarian at our institution also preferred using the ovine model for these reasons. Monkeys would likely be more extrapolative to humans but for obvious reasons, it is an expensive and difficult animal model. Pearce et al. [11] compared different animal models with respect to implant biomaterial research in bone and found the ovine model to be as acceptable as dogs for implant research.

The choice of the femoral condyle as the site for histomorphometric analysis was due to a number of reasons. Firstly, the sheep mandible can be a difficult model for dental implants as evidenced in previous research at the University of Otago. It is very hard and dense and there is difficulty in removal of teeth prior to implant placement. Secondly, the femoral condyle had ample cancellous bone and with its marrow space appeared more representative of type IV bone. The rabbit femoral condylar head has been

Table 1 Percent bone to implant contact (%BIC) of best 3 threads

	Control implant			Test implant		
	Implant1 %BIC	Implant2 %BIC	Mean %BIC	Implant1 %BIC	Implant2 %BIC	Mean %BIC
5 days	59.3 ± 24.7	85.6 ± 11.8	75.1 ± 22.0	47.3 ± 22.3	69.4 ± 17.0	56.1 ± 22.9
2 weeks	6.2 ± 17.9 [†]	64.8 ± 41.7	35.5 ± 43.4	35.6 ± 32.9	43.8 ± 18.9	39.7 ± 25.9
4 weeks	9.1 ± 9.2 [†]	21.5 ± 20.7	14.4 ± 16.2*	62.6 ± 15.8	67.8 ± 21.2	64.3 ± 17.3*
8 weeks	34.0 ± 19.8	32.3 ± 18.1	33.5 ± 19.1*	67.9 ± 17.1	74.2 ± 37.2	70.4 ± 26.5*
12 weeks	8.4 ± 13.3 [†]	46.6 ± 20.0	31.3 ± 25.8*	68.3 ± 22.2	69.2 ± 29.1	68.8 ± 26.0*
16 weeks	51.0 ± 23.5	42.1 ± 22.9	46.5 ± 23.1*	92.1 ± 10.3	82.0 ± 15.1	85.4 ± 14.2*

Six intervals ranging from 5 days to 16 weeks. %BIC of three best consecutive threads with standard deviation

Mean: average of 3 best consecutive threads from implant 1 and implant 2

* Observational differences noted at 4,8,12 and 16 week intervals

[†] Failure of osseointegration of 3 control implants: 2 weeks (implant 1), 4 weeks (implant 1) and 12 weeks (implant 1)

Fig. 5 Graph of Table 1. Best 3 threads average of per cent bone to implant contact (%BIC) of test and control implants at six different intervals. Red control, Blue test. (Color figure online)

Fig. 6 Rebound and formation of new bone of test and control implants over time

used extensively in research with regards to dental implants and the head Veterinarian at our institution was much more in favour of using sheep over rabbits since femur fracture

would not be an issue in sheep compared to rabbits. In addition, there was very little gait disturbance to the sheep and they returned to function quickly whereas eating can be impaired when implants are placed in the sheep mandible. Also, this was a pilot study to solely look at %BIC using sheep as their own controls.

Although the explanations for this are speculative, it is intriguing to think that keratin may deliver growth factors and/or cytokines as hypothesised by other authors. Sierpinski et al. [6] stated that keratin was “neuroinductive” which means it is possible that the same cellular or sub-cellular processes could be having an analogous effect of osseous healing and osseointegration at a cellular or sub-cellular level. Further histochemical examination of the keratin coated samples for osteoclast and osteoblast activity may be appropriate. For example, the keratin may have effects on osteoclast activity and further investigation along these lines would be interesting.

Three of the control implants demonstrated minimal osseointegration at 2, 4 and 12 weeks. Lack of osseointegration or failure of dental implants may be due to factors such as induced bone necrosis caused by excessive heating due to blunt burs, excess bur torque, lack of irrigation, the presence of dense bone or operator inexperience [12, 13] although the claim of implant failure due to operator inexperience has been challenged [14]. In this study these variables for failure were either controlled or equivalent between the two groups.

The results suggest that keratin either protected or buffered the osteotomy site from osteoclastic resorption perhaps by influencing osteoclast activity directly, or keratin accelerated the formation of new bone by osteoblasts possibly by inducing the transformation of undifferentiated mesenchymal cells into osteoblasts directly or by providing a scaffold to conduct new forming capillaries and their surrounding osteoblasts adjacent to the implant surface.

These suggestions remain exploratory since the cellular and sub-cellular processes of keratin on bone are as yet undetermined.

A meta-analysis of three implant studies performed by Duncan [15] reported a mean %BIC at 8 weeks of 59.4 % [SD 5.4 %] and 68.1 % [SD 11.1 %] at 12 weeks. Of these, the highest %BIC at 8 weeks (63.6 %) was found adjacent to hydroxyapatite-coated titanium screws. These values were similar to the present results for test implants (70.4 %) but almost double the results for the control implant (33.5 %). Moroni et al. [16] have also published results combining femur and tibia sites in sheep, at 4 weeks healing the %BIC ranged from 30 % for machined surfaces to 54 % for hydroxyapatite surfaces and at 12 weeks from 24 to 61 % for the same surfaces. In this paper, the analysis included all implant threads within cortical bone, unlike the technique used in our study which was restricted to best-3 consecutive threads. Furthermore, the site within the sheep femur that we used had only trabecular bone and therefore would be expected to show lower %BIC compared to comparable time points within cortical bone noted in other studies.

Since keratin was unable to be electrochemically bonded to the titanium surface as per the plasma spraying of hydroxyapatite (HAP), the keratin was applied directly as a hydrogel in a similar manner to the experiment by Schopper et al. [17]. Our results confirm that the direct application of the keratin material was acceptable as a delivery method to the titanium-bone interface and complex bonding methods are unnecessary.

Keratin gel applied to Neoss bimodal-surface titanium implants at the time of surgery, appeared to enhance early healing (%BIC) at all intervals from 4 to 16 weeks compared with unmodified Neoss bimodal-surface control implants. Further trials using larger groups of animals are required to confirm these findings and should focus on healing intervals from 4 weeks onwards. This is the first report of the use of this keratin material around dental implants and these encouraging results suggest that keratin hydrogel may be beneficial for promoting osseointegration of dental implants.

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