RESULTS OF AN EXPERIMENTAL STUDY

Action of hyaluronic acid
on the wound healing process following extraction

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REMINDER

Hyaluronic acid stimulates the differentiation of granulation tissue during wound healing in the dental alveolus, and promotes alveolar bone consolidation following extraction.

Hyaluronic acid (HA) is a glycosaminoglycan of the general formula $(C_{14}H_{22}NO_{11})n$. It is an essential component of the extracellular matrix in connective tissue which is found in abundance in the alveolar environment. It is particularly dense in the superficial layers of the buccal mucosa where it contributes to the epithelial barrier effect at the same time as enhancing both the stability and elasticity of the peripheral connective tissue. When selectively broken down by hyaluronidase (an enzyme which is produced by bacterial plaque), the connective tissue extracellular matrix becomes disorganized leading to increased epithelial permeability. For decades now, its physiological virtues have made HA a valued therapeutic agent in fields as diverse as dermatology (1), ophthalmology (2), ENT (3), orthopedics (9) and, more recently, plastic surgery. It has no toxic effects and there are no contraindications to its use. Its activities against inflammation and infection together with its capacity to promote wound healing have meant that its uses have been extended to include dentistry for the treatment of inflammation and lesions in the mouth (6).
A pilot study in rabbits showed that, after tooth extraction, treatment of the alveoli with 0.2% HA resulted in enhanced clot formation and, at a later stage, accelerated breakdown of the clot and its replacement by granulation tissue. In this situation, faster differentiation of granulation tissue could speed up wound healing in dental alveoli and cut down the amount of time before implantation can be considered following tooth extraction.
The purpose of this study was to use histological analysis to evaluate the effects of treatment with a preparation containing 0.8% HA on post-extraction alveolar healing in rabbits.

Key Words
➢ Post-extraction
➢ Hyaluronic acid
➢ Wound healing
➢ Histomorphometry

MATERIALS AND METHODS

In a group of 15 rabbits (mean weight = 2.8 kg), the first mandibular molars on both sides were extracted. On the control side (assigned randomly), the evacuated alveolus was simply sutured with Vicryl 4/0 without any other postoperative treatment. On the other side, the experimental alveolus was filled with a hyaluronic acid-containing gel (Gengipro®, Groupe Acteon, Mérignac, France) which was introduced by syringe before closure of the wound (using the same kind of sutures as in the control animals).
The animals were administered a fluorochrome by intraperitoneal injection (calcein, 30 mg/kg body weight, Merck) to mark the mineralization front and to give a measure of the rate of bone apposition during alveolar consolidation. Labelling was performed 48 hours after extraction and then again after 12 and 19 days.
Groups of three anesthetized rabbits were sacrificed at different time points (3, 7, 13, 20 and 30 days) after extraction so that the wound healing process could be monitored over time. At each time point, specimens prepared from two of the three animals (chosen randomly) were fixed in 10% buffered formalin, demineralized in acid solution, alcohol-dehydrated, embedded in paraffin and sectioned in the vestibulo-lingual plane to generate sets of serial sections of 5-6 µm in thickness. These sections were prepared for analysis with Masson's Trichrome stain. Specimens from the third animal were fixed in 70° alcohol and embedded in resin (Heraeus Kulzer, Wehrheim, Germany) without prior demineralization. Sectioning in the vestibulo-lingual plane resulted in two or three sections of a thickness of about 30 µm for examination in a fluorescence microscope. Analysis of all the sections was carried out in masked mode and yielded a chronological, comparative description of wound healing in both control and HA-treated alveoli.
Using an image analysis system (Bersoft Images Measurement®), the following values (based on numbers of pixels) were derived from digitally recorded micrographs:
➢ total alveolar area (S)
➢ area occupied by newly formed bone tissue in the alveolus (s)
➢ s/S = % of alveolus filled with newly formed bone tissue
➢ bone apposition rate (BAR), i.e. the space filled between two sequential labelling operations as a function of the time interval.
All the measurements derived from the serial sections corresponding to the central third of the alveolus were taken together to generate mean results at each stage, and then used for a statistical comparison of control and HA-treated sites using Student's "t" test and the Wilcoxon non-parametric test, both applied to matched series of small samples.

Results

Descriptive analysis

Despite the practical difficulties associated with the procedure, extraction did not lead to any complications in any of the rabbits other than some inhibition of feeding (which in no case lasted more than 48 hours).
Figure 1 shows the chronology of the wound healing process in both types of site at each stage.
➢ On day 3, the alveoli were filled with a blood clot loaded with various types of blood cell suspended in plasma. The clot appears less uniform and more compartmentalized at the control sites (Figure 2).
➢ By day 7, the clot is already being broken down with a fine network of fibrin emerging (Figure 3). This hemolysis is mediated by polymorphonuclear cells and lymphocytes, and is progressing from the periphery and the base of the alveolus towards the center. Metabolism of the clot is associated with classic osteoclastic resorption at the base of the alveolus (Figure 4). Hemolysis has proceeded more rapidly at control sites, where granulation tissue is already occupying a large part of the alveolus (Figure 1/4). There is even evidence of filling at the apex with fine, nascent bridges crossing hypervascular spaces. Fragments of fibers torn out during extraction are still seen anchored to the alveolar wall (Figure 5).

➢ On day 13, the next stage is characterized by frank regression of the blood clot which has been replaced with granulation tissue containing a high density of fibroblasts, collagen and vessels. This granulation tissue is already beginning to differentiate into bone at the base of all the alveoli and is extending up the sides at HA-treated sites (Figures 1/5 and 1/6). At the same time, a dense network of neovessels is developing (Figure 6). A few fragments of bone torn out of the alveolar wall during extraction are now being actively resorbed (Figure 7).
➢ By day 20, bone remodelling activity has intensified producing highly vascular, trabecular bone which has accumulated very quickly in the cervical direction. Differences in the degree of filling of the two different types of site are still discernible with the cervical third of control sites remaining relatively undifferentiated (Figures 1/7 and 1/8).
➢ On day 30, the dense vascular network in the newly formed bone at control sites reflects slower maturation there (Figure 1/9) compared with the HA-treated sites. The difference between control and experimental sites with respect to filling is less marked and the amount of bone at all sites is slightly reduced compared with the initial level.

Histomorphometric analysis

The results are summarized in Table 1. Area measurements (S and s) are expressed in relative values without correction for the magnification factor. Only the data for stages at which bone remodelling was detected (days 7, 13, 20 and 30) are shown, making a total of 8 animals (2 per stage). In these circumstances, statistical tests designed for matched series from small samples were used with each animal acting as its own control. Since these tests are based on differences observed between control and experimental sites at each stage, there was no point in testing for significant differences between time points since this is implicitly covered in the overall test.

A significant difference ($p < 0.05$) was observed with respect to alveolar filling between control sites (36.2 ± 31.2%) and HA-treated sites (60.4 ± 35.0%). Time point analysis showed that bone deposition could begin very early after HA treatment (as of day 7), and that it subsequently increased at a higher rate than at untreated sites. Later, towards the end of the healing process, the difference was less marked (Figure 8). The bone apposition rate (BAR) was higher in HA-treated sites, especially around day 12 and to a lesser extent by day 19 (Figures 9 and 10). Although this parameter was only measured in a single animal at each time point, the results are entirely consistent with those of the other analyses.

Discussion

The results presented here confirm those of a pilot study conducted in the same animal model but with a preparation containing one-quarter the concentration of HA. Thus, HA appears to promote wound healing and bone consolidation following tooth extraction.
In a more general way, the ability of HA to promote wound healing has been demonstrated in a variety of situations.
HA has been shown to promote the migration and maturation of keratinocytes in mucosal re-epthelialization (7, 10), and it has even been proposed as a possible marker for effective healing in soft tissues prior to implantation (4). Its activity is manifest in granulation tissue where it is abundantly produced and counters the damage induced by reactive oxygen intermediates (11). In the results presented here, the presence of HA in the alveoli appeared to promote and accelerate replacement of the blood clot with granulation tissue in that this phenomenon occurred earlier at HA-treated sites than it did at control sites.

Its physico-chemical properties mean that exogenous HA is readily incorporated into the extracellular matrix where it promotes interactions between the various molecular species which compose this ground substance. Apart from structural considerations, HA's biological activities include control of the migration and differentiation of various cells through receptor-dependent mechanisms regulating gene expression (1). This activity vis-à-vis cellular metabolism has been demonstrated by direct HA treatment following pulpar amputation, which strategy promoted the differentiation of odontoblasts and the deposition of reparative dentine (8). Members of another polysaccharide family—the chitosans, the structure of which is similar to that of HA—have been shown to promote the differentiation of immature bone cell progenitors and the formation of new bone tissue in tissue culture (5). These *in vitro* observations have been reproduced *in vivo* using an esterified form of HA (3) which stimulated the formation of new bone on the ventral and dorsal sides of the murine calvaria, an observation which led the authors to posit the osteogenic potential of HA. **In the course of alveolar wound healing in rabbits, this potential manifests by the early differentiation of granulation tissue into osteogenic, mesenchymatous blastema**, followed by the deposition of newly formed bone tissue as of day 7 after the insult. In the experiment reported here, HA-treated sites maintained their lead over control sites throughout the 30 days of the study. Comparable results have been reported following femoral trephination in rats (9): HA-treated lesions were filled in half the time that it took to fill control lesions.
In conclusion, in line with previous reports, the results presented here indicate that HA promotes wound healing and bone consolidation following tooth extraction. This suggests that HA treatment could be used **to cut down the interval between extraction and implantation** as illustrated in Figure 11. To confirm these preliminary encouraging results, studies of larger populations are necessary.

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TAKE-HOME MESSAGE

The aim of this study was to investigate wound healing and bone deposition following tooth extraction and treatment with a preparation containing 0.8% hyaluronic acid (HA) by comparison with control animals not treated with HA. The wound healing process was monitored in the alveoli by histological analysis of specimens taken from 15 rabbits at various time points (3, 7, 13, 20 and 30 days after extraction). Breakdown of the blood clot and its replacement by granulation tissue occurred earlier in HA-treated sites, as did the differentiation of granulation tissue into osteogenic blastema and the filling of the alveolar space with newly formed bone tissue. If these results can be confirmed, HA—by virtue of its ability to promote wound healing and bone formation—may prove to be a valuable treatment modality to reduce the interval necessary between tooth extraction and implantation.

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TABLES & FIGURE LEGENDS
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Table 1 – Histomorphometric data

		Control	HA
Alveolar area (S) (pixels)	Day 7 (n = 2)	101,941	94,003
	Day 13 (n = 2)	103,802	68,428
	Day 20 (n = 2)	94,620	110,245
	Day 30 (n = 2)	108,464	105,623
Area of newly formed bone (s) (pixels)	Day 7	0	9,136
	Day 13	25,327	44,557
	Day 20	45,312	88,818
	Day 30	7,914	91,268
Alveolar bone consolidation (s/S) (%)	Day 7	0	5.7
	Day 13	24.4	65.1
	Day 20	47.8	80.5
	Day 30	72.8	86.4
	mean	36.2 ± 11.2	60.4 ± 35

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(à droite, verticale) Control sites HA-treated sites

day 3 day 7 day 13 day 20 day 30

1. Reconstitution of the chronology of wound healing at control sites and HA-treated sites between day 3 and day 30 (Masson's Trichrome stain; magnification X8):
1/1 to 1/3 The clot fills the alveolus
1/4 Granulation tissue has replaced a large part of the clot. The base of the alveolus is already partially filled (><)
1/5 Only the base of the alveolus is filled.
1/6 The filling is beginning to climb the alveolar walls (>)
1/7 to 1/10 Bone consolidation is continuing in the cervical direction. The sites treated with HA (1/8 and 1/10) are still further advanced than the control sites

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2. Control site on day 3 entirely filled with a clot which is apparently compartmentalized (Masson's Trichrome stain; magnification X100)

3. Control site on day 7. The granulation tissue si progressively replacing the clot which is being broken down (Masson's Trichrome stain; magnification X100)

4. Control site on day 7. The alveolar wall is undergoing osteoclastic resorption (arrows) (Masson's Trichrome stain; magnification X250)

5. HA-treated site on day 7. Fiber fragments remain attached to the bone (arrow) (Masson's Trichrome stain; magnification X250)

6. HA-treated site on day 13. An active remodelling process is underlying alveolar consolidation (Masson's Trichrome stain; magnification X250)

7. HA-treated site on day 13. Bone fragments torn out of the alveolar wall are being resorbed (arrows) (Masson's Trichrome stain; magnification X100)

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8 Alveolar bone consolidation (%) between day 7 and day 30

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9. HA-treated site. the location of the mineralization front is shown on day 2 (white arrow) and day 12 (red arrow) (magnification X36)

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10. Bone apposition rate (mm/100) based on fluorochrome staining

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11. Clinical observation
a Image of the alveolus after extraction of 41 and filling using Gengipro®
b Monitoring image one month after surgery
c Implant two months after extraction