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Microradiography to evaluate bone growth into a rat mandibular defect

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Summary Microradiography has been evaluated to measure bone healing into a 5.0 mm outer diameter mandibular defect in the rat. This method provides high-resolution radiographs of the defects that can be used for an accurate measurement of bone defect healing. In 12 rats, the defect widths of 42-day-old mandibular defects have been measured both using microradiographs and histological sections. The defect width ± S.D. measured 3.42 ± 0.98 mm microradiographically and 3.47 ± 1.11 mm histologically. Both methods were accurate in determining defect widths but microradiography has the advantage over histology that an image is obtained from the entire defect, making it possible to measure areas of bone growth.

Introduction

Animal models are often used to evaluate new ways of inducing or influencing bone growth. In the maxillofacial skeleton, a frequently used animal model is the mandibular 'critical size' defect in the rat.[1,2] This model consists of a circular through-and-through defect of a diameter varying from 4 to 7 mm drilled into the mandibular ramus. The term 'critical size' implies that the defect will not heal spontaneously,[3–5] so that healing, if obtained, is caused by the experimental intervention. The rat mandibular defect model has been used to evaluate ingrowth of bone substitutes[1,2] and osteoconductive properties of membranes with[6–10] or without[11,12,13] growth-stimulatory factors. To evaluate the treatment effect, bone growth inside the defect traditionally has been measured histologically using sections through the center of the defect. Although histological evaluation of bone growth inside the defect is considered the 'golden standard', there are limitations to this technique. An important limitation is that the histological section represents one specific area of the defect, which does not necessarily represent the entire defect. Furthermore, the diversity in histological scoring systems makes comparison between studies difficult.

Quantitative microradiography is a commonly used technique to measure mineral distributions (calcium, phosphate) and mineral amounts of carious lesions in enamel and dentin.[14,15] The technique has also been used to measure mineral distributions in bone.[16–18] It provides high-resolution radiographs, which may also be used to provide a better overall picture of bone growth into a mandibular defect. To evaluate this, defect widths in 42-day-old rat mandibular defects were measured using both

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microradiography and histology, and subsequently, the techniques were compared to each other.

Materials and methods

The study protocol was approved by the Animal Studies Review Comittee, and in accordance with Institutional Guidelines (University of Groningen, The Netherlands).

Operative procedure

In 12 rats (Sprague—Dawley, male, age 15—17 weeks, mean weight ± S.D. 350 ± 13 g, range 330—367 g) a standardized 5.0 mm circular mandibular defect was created into the right ramus of the mandible: Under nitrous—oxygen—halothane inhalation anesthesia, the mandibular and hemicervical areas were shaved. After disinfection of the skin, a submandibular incision was made and the masseter muscle was exposed. After incision of the muscle along the submandibular border, a muscle flap was raised on the buccal and lingual side. Care was taken not to injure the facial nerve and parotid duct. Using a 5.0 mm outer diameter trephine drill (22RF050, Meisinger, Germany) mounted in a dental technician drill, a through-and-through defect was drilled into the mandibular ramus (Fig. 1). During drilling, the surgical field was continuously irrigated with saline to reduce thermal damage. After the defect was drilled the wound was rinsed with saline. Subsequently, the wound was closed in layers using 4—0 resorbable sutures. For postoperative pain relieve, a single dose of buprenorphine 0.03 mg/kg was given. The rats were housed in groups, and received standard laboratory food and water ad libidum. After 42 days, the rats were anesthetized by inhalation anesthesia and sacrificed by an intracardial injection of an overdose pentobarbital. Subsequently, the right mandibular half was explanted and fixed in phosphate-buffered formaline solution. After 48 h, the specimens were rinsed with saline and put in 70% denatured ethanol solution. Excess of muscle was removed from the specimens by means of a scalpel.

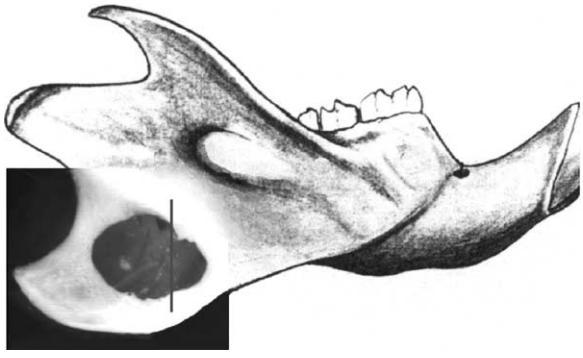

Figure 1 Schematic representation of right side of the rat mandible. The location of the defect is represented by a superimposed microradiograph of a 42-day-old mandibular defect. The vertical line represents the place where the histological section has been made (Fig. 2).

Microradiography

An X-ray source (Philips PW 1730, The Netherlands) was used that produced monochromatous radiation with a specific wavelength of 1.537 Å. The X-ray radiation used is Cu Kα radiation with a Cu X-ray tube and a nickel filter. The wavelength produced is especially sensitive to be absorbed by calcium. The explanted parts of the mandible were placed between the 35-mm film (Fuji B&W POS/71337) and the X-ray source and exposed for 25 s, with a tube charge of 25 kV and 25 mA. Care was taken to place the plane of the defect parallel to the film. To minimise magnification effects, the distance was kept small (0.3 mm) between the specimen and the film and large (300 mm) between the X-ray source and the specimen. Film was used instead of radiographic plates because of a much higher resolution of the film. After development of the film with a D-19 developer (Kodak) for 10 min, fixating, rinsing, and drying, the film was placed on a light box. A digital image of the mandibular defect on film was recorded with a stereo microscope (Wild/Leitz M7 S, Switzerland) with a magnification of 10× and a CCD camera (Teli CS 8310, Tokyo, Japan). The camera was linked to a personal computer equipped with a framegrabber. The magnified microradiographs were stored as images with a size of 640 × 480 pixels and with a resolution of 256 gray values. In addition, a separate image of a microruler was recorded for calibration (Fig. 2A).

Histology

The mandibles were dehydrated in series of ethanol and embedded in methylmethacrylate without decalcification under negative pressure. After the middle of the defect had been determined by placing the mandible imbedded in PMMA on top of the corresponding radiograph on a light box, the specimen was sawn into halves. Sections of 4-μm thickness were cut at the cutting edge from one-half of the embedded specimen using a microtome (Jung-K, Heidelberg, Germany). The sections were stained according to the Goldner trichrome method. The histological sections were placed on a light box and

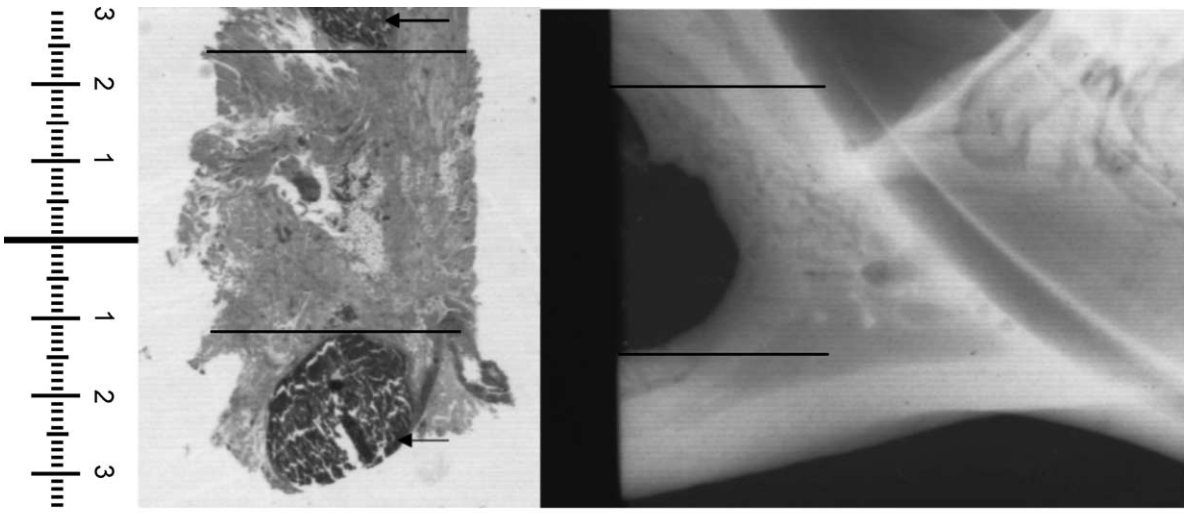

Figure 2 Bone defect width measured using image analysis software on both the histological section (B) and the corresponding microradiograph (C). Two horizontal parallel tangents were drawn at the inner bony edges of the defect. The perpendicular distance between these two lines was measured. A separate image of a microruler was used for calibration (A) (magnification: 10×). The arrows in (B) indicate bone.

digital images were recorded and stored in the same way as the microradiographs (Fig. 2B).

Comparison between microradiography and histology

After sectioning, a microradiographic image of the remaining imbedded part of the mandible was made (Fig. 2C). The cutting edge of this radiograph exactly shows where the last histological section was made. Both on the histologic specimens and the microradiographs the defect width was measured using image analysis software (Scion Image, version beta 4.0.2). The image analysis software rather than direct measurement was used because extensive experience was already present using this convenient method. Parallel tangents were drawn at the defect rims and the perpendicular distance in number of pixels was measured automatically between these tangents. A defect rim was histologically defined as the most inner point of bone growth inside the defect. The distances measured in millimeters on the microradiograph and the corresponding histological sections were compared to each other. Each measurement on the histological section and on the microradiograph was repeated three times and then averaged.

Results

The surgical procedure was uneventful and all rats recovered well. No wound infection or dehiscence did occur. All animals gained weight. The defect widths as measured on the histological specimens and on the microradiographs are presented in Table 1. The pixel size measured 0.0172 mm^2. In one defect, the embedded specimen had been sectioned deeper from the cutting surface that had been histologically measured. In another defect, a very thin rim of bone could be histologically detected, but not on the microradiograph. Excluding the first case, the results show that the defect width $\pm$ S.D. as measured by histology (3.47 ± 1.11 mm) was 6.8% larger than that measured using microradiography (3.42 ± 0.98 mm).

Discussion

A new promising method of evaluating bone growth into the rat mandibular defect using microradiographs was described.

In two mandibles, the measurements could not be fully compared. In one defect, the embedded specimen had been sectioned deeper from the cutting surface that had been histologically measured. This means that the width as measured histologically did not represent the site where the width has been measured by microradiography. In the other case, a very thin rim of bone could be detected histologically, but not on the microradiograph. This rim was less than 0.09 mm thick. Excluding these two cases, the results show that the defect width as measured by histology was about 5% larger than the width measured using

Table 1 Defect width as measured by microradiography and histology.

Defect number	Mean defect width (mm) ± S.D. (mm)		Difference of means (mm)	Microradiography (%)
	Microradiography	Histology		
1	2.69 ± 0.03	3.89 ± 0.01	1.20	+44.6[a]
2	3.97 ± 0.02	4.00 ± 0.01	0.03	+0.8
3	2.56 ± 0.01	2.67 ± 0.01	0.11	+4.3
4	3.47 ± 0.02	3.60 ± 0.01	0.13	+3.7
5	2.12 ± 0.01	2.45 ± 0	0.33	+15.6
6	1.81 ± 0.01	1.83 ± 0.01	0.02	+1.1
7	3.07 ± 0.03	2.07 ± 0.01	1.00	−32.6[b]
8	4.43 ± 0.01	4.73 ± 0.03	0.30	+6.8
9	3.02 ± 0.01	3.04 ± 0	0.02	+0.7
10	4.50 ± 0.02	4.51 ± 0.01	0.01	+0.2
11	4.41 ± 0.02	4.82 ± 0.03	0.41	+9.3
12	4.29 ± 0.01	4.47 ± 0.01	0.18	+4.3
Mean excluding 1[a]	3.42 ± 0.98	3.47 ± 1.11	0.23	+6.8

[a] The embedded specimen has been sectioned deeper after cutting the slice which has been histologically measured.
[b] This specimen showed histologically a very thin rim of bone (<0.09 mm thickness) growing inside the defect, which could not be detected on the microradiograph.

microradiography. An explanation for the consistent slightly larger dimensions as measured histologically may be due to artifacts in the preparation of the sections. As can be observed in Fig. 3, space is evident between the muscle fibers, indicating that the histological specimen probably has been torn during the preparation process. These preparation artifacts were seen to some extent in most of the sections. Due to this, the overall length is slightly larger than the original length (as measured by microradiography), which may account for the observed differences. However, it must be noted that the specimens may shrink during the dehydration process, which may counter the aforementioned increase in length. In any case, the results show that microradiography as compared to histology can accurately distinguish the bony edges of the defect.

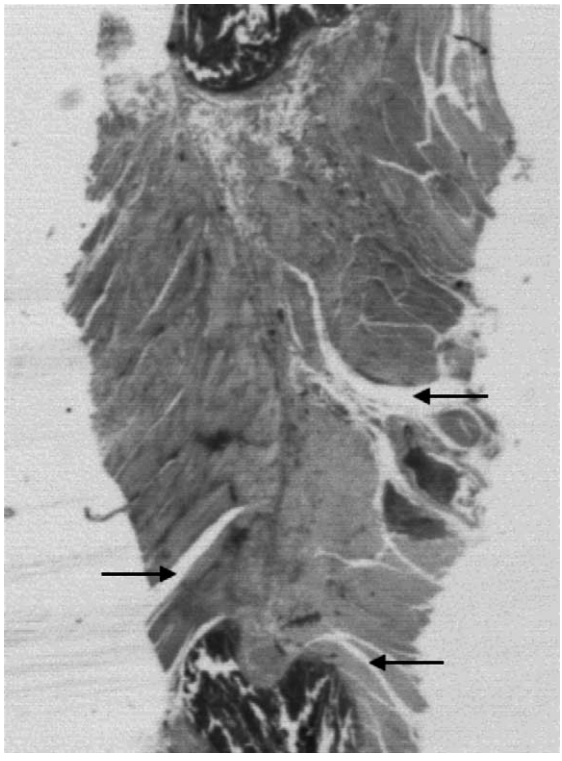

Figure 3 Histological section though a mandibular defect. Space is evident between the muscle fibers (arrows), indicating that the histological specimen has been torn during the preparation process (magnification: 10×).

Although histology is usually considered the 'golden standard' in evaluating bone healing in experimental defects, there seems to be no real 'standard' scoring system. Mostly, a modification of Heiple's[19] semi-quantitative scoring system is used,[6,7,13,20] but these modifications differ from each other, making comparison between studies difficult. Furthermore, because bone growth inside a defect is more or less irregular, histological evaluation of bone growth using sections through the center of the defect[10,21] may not represent bone growth in other regions of the defect. Although evaluation of defect healing using conventional radiographs has been attempted, giving a more complete picture of defect closure, it was only scored semi-quantitatively, e.g. no, partial or complete healing/closure,[22,23] probably due to lack of radiograph quality.

Microradiography can provide a solution to the limitations of the present techniques in evaluating

experimental bone defect healing. The results show that bone boundaries can be detected with accuracy in the plane of the defect. This means that, using microradiographs, not only distances can be measured (one dimension), but areas of bone growth into the defects as well (two dimensions). This seems more appropriate in evaluating bone defect healing than measuring the diameter in the middle of the defect using histology. Furthermore, by providing high-resolution microradiographs in the plane of the defect, patterns of bone growth can be visualized. Another advantage of the microradiograph technique over histology is that the microradiographs can be easily obtained, in a relative short period of time, and at minimal cost.

Nevertheless, microradiography does not allow evaluation of bone growth on the cellular level (in contrast with histology), and only calcified tissue can be detected. In one case, a thin calcified bone rim (<0.09 mm) could not be detected on the microradiograph while it could be seen histologically. Despite the infrequent occurrence and the debatable significance of a very thin sheet of bone, it stresses that microradiography does have limitations in detecting bone. Also, although microradiography is capable of determining whether or not bone is present (qualitative), a lateral microradiograph does not allow calculating bone volume (quantitative) that is present in the defect.

Summarizing, microradiography has some apparent advantages in the evaluation of bone healing of experimentally created defects as compared to histology or conventional radiography. Thus, the microradiography technique seems promising to evaluate bone growth into defects that do not contain any radiopaque material. This is the case with bone morphogenetic proteins, growth factors and non-radiopaque osteoconductive membranes or tissue scaffolds. Future studies are needed to determine whether this technique can be applied to measure bone formation in defects when radiopaque material is present, such as bone grafts or bone substitutes. Although microradiography was evaluated on the rat mandibular defect, it seems that it can also be used in other animal bone defect models such as the calvarial[24] and the nasal defect.[25]

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