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EVALUATION OF SHAPE AND SIZE CHANGES OF BONE AND REMODELLED BONE SUBSTITUTE AFTER DIFFERENT FIXATION METHODS

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Suitable tissue fixation is indispensable to histological analysis. This investigation, therefore, sought to evaluate changes of shape and size of bone specimens and remodelled bone substitute material following different fixation methods. Mandibular bones of 9 pigs (*Sus scrofa domesticus*) served as specimens. Two mandibular premolars were extracted respectively and the extraction alveoli were filled with synthetic bone substitute material. The samples were collected after 70 days. Fixation of 6 specimens respectively was done for 7 days in 4% formalin (formaldehyde), 70% ethanol and glycerol at 18°C room temperature. The samples were radiographically examined before and after fixation using a reference specimen and subsequently underwent histological analysis. After fixation in formalin, the samples showed no size changes. After fixation in glycerol, morphological analysis revealed minor shape changes. Fixation in ethanol causes shrinking of the tissue specimens. Histological inspection of the tissues shows no morphological changes except slight shrinking. In conclusion there is no universal fixative that could meet all requirements and permitted proper examination without affecting tissues or bone specimens.

Key words: *bone substitute, remodelling, fixation, histology*

INTRODUCTION

The number of research projects in the scope of orofacial bone defects and bone replacement is continuously increasing (1-3). Frequently, the selection of analysis methods for determination of quantitative changes is particularly difficult (4, 5). Histological investigation is still the gold standard for the analysis of changes. Radiographic documentation may reveal numerous changes, however, is often insufficient for basic research. In particular, radiographic examination alone does not lend itself to the disclosure of cell changes. Histological findings, therefore, are further required in addition to radiographic examination (6).

Besides large defects, minor changes such as in the oral area are difficult to treat. Small defects, for instance in the alveolar ridge area, are an obstacle to prosthetic management as well as to orthodontic treatment. The presence of teeth or implants in the oral tissue specimen has to be expected during preparation (7). Since such analyses deal with complex structures, native preparations can only be produced rarely.

Histological processing of oral bone tissue always requires proper fixation (8). Fixation is meant to provide a snapshot of tissue conditions without alteration or damage. The possibility of false-positive histochemical reactions due to improper fixatives has to be considered. Furthermore, the quality of the fixed specimens may be affected. Especially during bone fixation, changes of the morphological structure can frequently occur. Fixation of bone specimens is particularly complicated following treatment with bone substitutes. The substitute materials are surgically implanted into the bone defects. After some time the bone substitute materials are to be fully remodelled by native bone (9). However, bone structure changes after remodelling. Such bone has different properties and may, therefore, show different reactions to fixatives.

Further problems during fixation and histological analysis may be posed by bone specimens containing residual bone substitute. Since bone substitute consists of calcium-containing material, it may be dissolved during fixation.

Formalin (formaldehyde) is the most frequently used chemical fixation medium. The health hazardous and unpleasant handling properties on the one hand and the fixation-related disadvantages of formalin on the other hand have repeatedly prompted the use of different fixatives. Besides formalin, ethanol and glycerol are used for specimen fixation. Ethanol is particularly advantageous for bone preparation due to its critical role in Technovit embedding.

This study aimed to evaluate the shape and size changes of mandibular bone specimens with different fixatives. The bone to be analyzed was used with bone substitute before fixation. The bone substitute was implanted in the extraction wound and fully remodelled into new bone.

MATERIAL AND METHODS

The approval number of the animal trial by the local Ethics Committee is LVL M-V/TSD/7221.3-1.1-037/05.

Sample fabrication

Specimens were sampled from pigs ("Deutsches Landschwein", *Sus scrofa domesticus*). Nine animals were operated and investigated for this purpose. The pigs were randomized. Each animal was kept in a box of 16 m² in size.

The two mandibular premolars were extracted bilaterally and the alveoli were filled with bone substitute material respectively. In order to minimize animal stress, extraction and insertion of bone substitute were carried out under short-term anaesthesia. Premedication using ketamine (10 mg/kg body weight i.m. in the *M. semitendinosus*) (B. BRAUN, Germany), was followed by a combined anaesthesia with 150-200 mg ketamine, 4.0-7.0 mg Droperidol and 4.0 mg Faustan i.v. in the *V. auricularis caudalis*. The extraction alveoli of the right side were provided with NanoBone® immediately postsurgery. Wound closure was done using a mucoperiosteal flap. Total treatment time was about 20-25 minutes.

After 70 days, the specimens were collected under anaesthesia without revival of the test animals.

Specimen preparation, fixation and radiological examinations

Immediately after collection, the specimens were reduced to a minimum. The adjacent muscles and soft tissues were resected. Subsequently, the bone structures no longer required were removed. To demonstrate the changes occurring during fixation, reference specimens were permanently implanted into the bone. Implantation was performed on the right and left side of the alveolar defect in the tooth region. The distance between the reference specimens was 13 mm. Mini-screws (Mondeal, LOMAS Screw 1.5x9 mm, Germany) were used to ensure standardized implantation and bone loading (7). Prior to fixation in formalin, ethanol and glycerol the samples were radiographed using an implanted reference specimen. Control radiographs were also obtained after fixation and embedding. The radiographs were taken using an Orthophos XG apparatus (Sirona, Bensheim, Germany). The film-focus distance was 1.5 m. The standard program C4 (64 kV at 16 mA, 9.1 s) was used for examination.

Fixation of 6 specimens respectively was performed after Romeis (10) in 4% formalin (11), 70% ethanol and glycerol at 18°C room temperature for 7 days (10). The respective fixation times depended upon specimen dimensions. The diffusion speed of formalin, ethanol and glycerol was determined to be 1mm per hour (12).

All specimens were sufficiently fixed with all methods to enable conservation and processing. The specimens were re-radiographed after fixation, and the results were compared to the initial state.

For fabrication of the Technovit 9700 (Kulzer GmbH, Wehrheim, Germany) blocks, the fixed bone samples were further prepared by dehydration in an ascending alcohol series.

Statistics

The graphical presentation and the measurement values given in *Table 1* represent arithmetic means with standard deviations. Deviation from expected values was tested for significance using Student's t-Test. Statistical analysis was done using the SPSS for Windows (ver. 10.02) software.

RESULTS

After tooth extraction and alveolar filling with bone substitute the animals' body weight developed normally. The weekly clinical checks failed to reveal

disturbed intraoral wound healing. Thus, all animals were eligible for sample collection. Since the extraction wounds are considered minimal, the amount of bone substitute implanted was completely remodelled as early as after 70 days.

Radiological measurements

After formalin fixation, the samples showed slight shape changes due to shrinkage by about 4% (significance $p < 0.010$) as compared to controls. Markedly larger changes were noticed after specimen fixation with glycerol (8% shrinkage) and ethanol (11% shrinkage). These changes were significant as well compared to the control values ($p < 0.005$) (Table 1).

Table 1. Comparison of specimen shrinkage in per cent. T1- Fixation of specimens after preparation at 18°C room temperature for 7 days. T2- dehydration of bone samples in an ascending alcohol series. T3- After fabrication of the Technovit 9700 blocks.

	formalin	ethanol	glycerol
T1/T2	0.96%	0.89%	0.92%
T1/T3	0.90%	0.87%	0.88%
T2/T3	0.95%	0.97%	0.96%

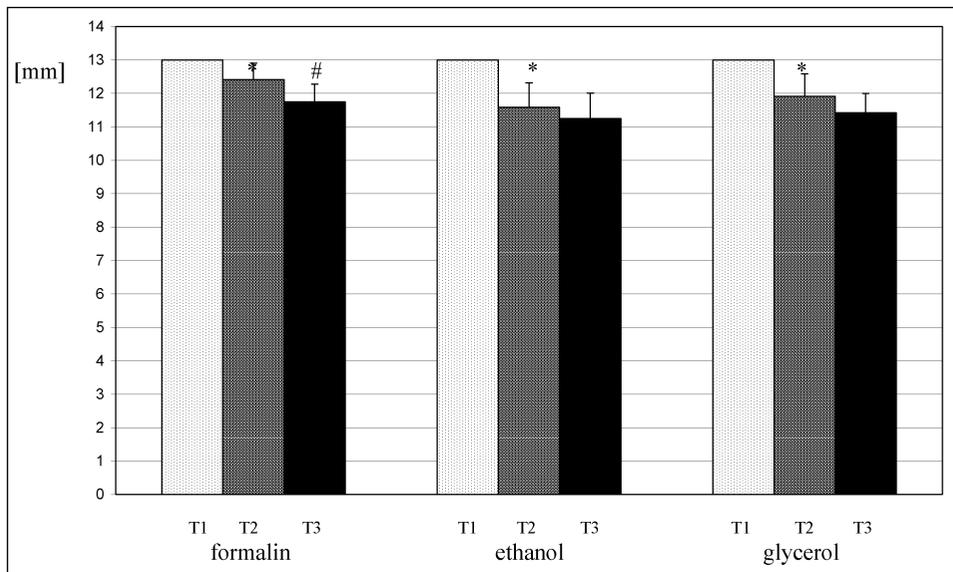


Fig. 1. Shrinkage (in mm) in different fixatives. T1- Fixation of specimens after preparation at 18°C room temperature for 7 days. T2- dehydration of bone samples in an ascending alcohol series. T3- After fabrication of the Technovit 9700 blocks.

* significant between T1/T2 ($p < 0.05$); # significant between T2/T3 ($p < 0.05$)

Also, the changes varied among the fixatives. A significant difference was observed between formalin and ethanol ($p < 0.05$).

Techovit embedding caused additional, yet slight changes of specimen size. Due to further dehydration, almost all specimens shrank evenly without significant differences.

After curing of Technovit the specimens had shrunk by about 10% in formalin, about 13% in ethanol and 12% in glycerol. All types of fixation and subsequent embedding led to specimen shrinkage (*Fig. 1*).

Histological analysis

Histological analysis of the specimens permitted assessment of individual cells and size comparisons with all types of fixation. Histological analysis also allowed assessment of bone remodelling. A particularly thick compact layer was noticed in the remodelled areas of all samples.

In contrast to the other fixatives, specimen fixation with ethanol induced formation of a “crust“ which cannot be seen but histologically. This hardening of the outer regions prevents further rapid penetration of the fixative.

DISCUSSION

Bone histology is indispensable to the assessment of bone remodelling after implantation. However, fabrication of bone specimens is somewhat compromised. Important criteria of successful therapy involve shape and structure of the native and the newly formed bone as well as the presence of residual bone substitute after treatment (13, 14). Unfortunately, histological analyses do not consistently provide a sufficient answer to this question. The present study, therefore, evaluates different fixation methods of native bone and bone newly formed from bone substitute. Preceding radiographic analysis of the specimens enabled optimum demonstration of deformed structures. Our preparatory work showed the advantages of micro-CT for the study of bone/bone substitute properties (15).

Histological processing of the specimens is required for demonstration of cellular changes. All methods evaluated provide a good opportunity for fixing bone specimens. However, minor morphological size changes occur which may cause a distortion of results due to variable fixation (16).

Optimum preparation of the specimens plays a crucial role for fixation (17, 18). The size of the tissue samples must be reduced to a minimum prior to fixation. The tissue should be trimmed according to the diffusion speed of the fixative. In the present study, we chose to resect the adjacent muscles and soft tissue to reduce specimen size. Improved perfusion of tissue liquids and lesser changes occur in small specimens (19). Tiny specimens also allow for the use of other diagnostic methods. To give an example, small samples are frequently frozen for rat trials in order to obviate possible shape changes.

Jaw specimens involving teeth and the newly formed compact layer which is particularly thick require fixing for further processing. A native section cut through existing teeth and the thick compacta may affect the entire specimen. Mainly the analysed structures between the teeth may undergo loading-related changes or destruction. The spongiosa is particularly delicate during processing. Pretests involving unfixated specimens revealed a strong impact of preparation on spongy bone and the interface to compact bone.

The fixation of dense bone is particularly difficult (17). A thick compact layer negatively affects the quality of the deeper regions. The autolytic processes continue in those regions not yet reached by the fixative. The centre of the specimen long remains unfixated especially during fixation of dense bone such as after treatment with bone substitute.

On the other hand, fixation alters the physico-chemical structure of bone tissue (12). Besides dehydration, a loss of minerals and proteins from the specimen occurs irrespective of the fixative. Through replacement of water molecules, the fixation process essentially preserves cellular details and prepares the tissue for embedding (10, 12). Replacement of bound water molecules in the immediate vicinity of mineral and collagen molecules by fixatives is impossible (12). Formaldehyde rapidly penetrates the tissue and binds primarily to proteins, while cross-linking is considerably slower. In contrast, ethanol is capable of rapid penetration, but as well induces marked "crust" formation and shrinking of the specimens. Alcohol removes the hydration shell of proteins resulting in coagulation with breaking of the hydrogen bridges and destruction of the tertiary structure. However, the chemical composition, also the amino acid sequence, is preserved. Due to its rapid dehydration effects, ethanol is poorly suited for large tissue blocks. The tissue becomes brittle and is unfavourable for cutting. Therefore, larger tissue blocks undergo preparation in fixative mixtures.

Our results show that ethanol fixation resembles glycerol fixation. These findings correspond to the reports from literature (12, 16, 18). The pH value of the fixing solutions is important in this context. It should be kept within the physiological range of 7.2 to 7.6 for aldehydes to ensure optimum reactions. The addition of buffers, *e.g.*, phosphate, TRIS, veronal or acetate buffers (electron microscopy: *e.g.* cacyl buffer) serves for this purpose. A 10% buffered formalin solution (besides formalin, the solution contained sodium barbital, glucose and sodium chloride) allows uniform fixing of the thin bone slices until further processing.

There is no universal fixative that meets all requirements and permits detection without affecting tissues. The present investigations are novel, as no such comparison of fixations exists in the literature.

With chemical fixation, specimen preparation has to be kept as small as possible. To ensure rapid tissue perfusion, a favourable surface/volume *ratio* of the samples is required. Formalin remains the fixative of choice for evaluation of histological bone specimens. Tests involving alternative substitute fixatives

have also yielded satisfying results, however, tissues are likely to be affected more strongly.

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REFERENCES

1. Bayerlein T, Proff P, Heinrich A, Kaduk W, Hosten N, Gedrange T. Evaluation of bone availability in the cleft area following secondary osteoplasty. *J Craniomaxillofac Surg* 2006; 34: 57-61.
2. Mai R, Hagedorn MG, Gelinsky M et al. Ectopic bone formation in nude rats using human osteoblasts seeded poly(3)hydroxybutyrate embroidery and hydroxyapatite-collagen tapes constructs. *J Craniomaxillofac Surg* 2006; 34: 101-109.
3. Proff P, Bayerlein T, Fanghanel J, Gerike W, Bienengraber V, Gedrange T. The application of bone graft substitutes for alveolar ridge preservation after orthodontic extractions and for augmentation of residual cleft defects. *Folia Morphol (Warsz)* 2006; 65: 81-83.
4. Czesnikiewicz-Guzik M, Konturek SJ, Loster B, Wisniewska G, Majewski S. Melatonin and its role in oxidative stress related diseases of oral cavity. *J Physiol Pharmacol* 2007; 58: 5-19.
5. Gonciarz M, Wloch M, Gonciarz Z. Helicobacter pylori in liver diseases. *J Physiol Pharmacol* 2006; 57: 155-161.
6. Gedrange T, Hietschold V, Mai R, Wolf P, Nicklisch M, Harzer W. An evaluation of resonance frequency analysis for the determination of the primary stability of orthodontic palatal implants. A study in human cadavers. *Clin Oral Implants Res* 2005; 16: 425-431.
7. Gedrange T, Proff P, Bayerlein T, Landsberger P, Dietze S, Fanghanel J. Histological and fluorescence microscopic examination of the bone/implant interface in orthodontic miniscrews (Mondeal). *Folia Morphol (Warsz)* 2006; 65: 70-71.
8. Rowlands DC, Ayres JG, Crocker J. The effect of different fixatives and length of fixation time on subsequent AgNOR staining for frozen and paraffin-embedded tissue sections. *Histochem J* 1993; 25: 123-132.
9. Bienengraber V, Gerber T, Henkel KO, Bayerlein T, Proff P, Gedrange T. The clinical application of a new synthetic bone grafting material in oral and maxillofacial surgery. *Folia Morphol (Warsz)* 2006; 65: 84-88.
10. Romeis B. Mikroskopische Technik. Munchen, Wien, Baltimore Urban & Schwarzenberg, 1989.
11. Lillie RD. Histopathologic Techniques and Practical Histochemistry. New York: Blankiston Co. Inc. 1954.
12. Yeni YN, Yerramshetty J, Akkus O, Pechey C, Les CM. Effect of fixation and embedding on Raman spectroscopic analysis of bone tissue. *Calcif Tissue Int* 2006; 78: 363-371.
13. Bayerlein T, Proff P, Fanghanel J et al. Wound management after the application of bone grafting substitutes in the orofacial region. *Folia Morphol (Warsz)* 2006; 65: 89-91.
14. Gedrange T, Bourauel C, Kobel C, Harzer W. Simulation of bone strain by orthodontic implants using the finite element method. *Biomed Tech (Berl)* 2003; 48: 287-290.
15. Gedrange T, Mai R, Weingaertner J et al. Finite element representation of bone substitute remodelling in the jaw bone. *Biomed Tech (Berl)* 2008; 53: 220-223.
16. Burck HC. Histologische Technik. Stuttgart, Thieme Verlag, 1982.

17. Lauer G, Haim D, Proff P et al. Plate osteosynthesis of the mandibular condyle. *Ann Anat* 2007; 189: 412-417.
18. Mai R, Lauer G, Pilling E et al. Bone welding—a histological evaluation in the jaw. *Ann Anat* 2007; 189: 350-355.
19. Proff P, Gedrange T, Franke R et al. Histological and histomorphometric investigation of the condylar cartilage of juvenile pigs after anterior mandibular displacement. *Ann Anat* 2007; 189: 269-275.

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