ORIGINAL PAPER
PŮVODNÍ PRÁCE

Staining Undecalcified Bone Sections a Modified Technique for an Improved Visualization of Synthetic Bone Substitutes

Barvení nedekalcifikované kosti – modifikovaný postup pro lepší vizualizaci kostních náhrad

U. MAYR-WOHLFART[1], G. RAVALLI[2], K. P. GÜNTHER[3], S. KESSLER[4]

[1] Institute of Transfusion Medicine and Immunogenetics, Ulm, Germany
[2] Orthopaedic Department, University of Ulm, Ulm, Germany
[3] Orthopaedic Department, University of Dresden, Dresden, Germany
[4] Orthopaedic Department, Sindelfingen District Hospital, Sindelfingen, Germany

SUMMARY

We describe a detailed embedding procedure for large bone spicemens in methyl methacrylate and a new staining method by which thin sections (appr 100 um) of undecalcified bones with synthetic implants can be coloured. Different staining effects were obtained which greatly facilitated the evaluation of sections with bone, new forming bone and especially remnants of synthetic implants. The identification and quantification of the latter is difficult in common staining techniques.

A detailed embedding – staining – mounting procedure is proposed.

Key words: bone histology, hard tissue histology, light mircroscopy, biomaterials, synthetic implants, staining, undecalcified sections.

INTRODUCTION

Histological and histomorphometrical examination of hard tissues using light microscopy techniques requires a standardized thin section and staining procedure. Although several methods have been proposed (1, 2, 5), undecalcified embedment of large bone spicemens especially in combination with synthetic bone substitutes is still challenging. Problems already can arise within the sawing and grinding process. Bone formations and embedding materials are much faster abraded than the more resistant synthetic substitutes, leading to sections with a marked surface roughness.

Major problems can arise, when staining of different calcified tissue components within one section leads to similar appearance of the components included. E.g. using the Masson Goldner staining technique (3), new bone formation is difficult to differentiate from bone of the host side as well as from remnants of bone substitute materials. A quantification of degradation rates of synthetic materials becomes difficult and less reliable.

It is the purpose of this short technical note to describe our embedding process and to present a new staining technique, which strongly allows the differentiation of synthetic calcified substitutes in contrast to new bone formation.

MATERIAL AND METHOD

Within a weight-bearing animal model, the histomorphological and histomorpho-metrical appearance of a neutralized glasceramics, called GB9N, (BIOVISION GmbH, Ilmenau, Germany) was tested. GB9N was implanted in the medial tibial head of adult Merino sheep 3 milimeters under the articular surface. Specimens were harvested four weeks after implantation and prepared for histomorphological and histomorphometrical investigations.

Preparation of Specimens

The explants were fixed with 4 % formalin (3) at least 5 days after removal from the body. The following processing schedule is suitable for biopsy size specimens which are processed in a 275 ml glass vial. The bone blocks were first dehydrated in graded alcohol (60% methanol 3 days, 80% methanol 3 days, 100 % methanol 3 days) and then defatted (methanol/acetone 1:1, 1 day; 100% acetone, 1 day; methanol/acetone 1:1, 1 day, 100 % methanol, 1 day) all at room temperature. Before embedding, transform the blocks into smaller glass vials (50 ml).

The embedding procedure is described as follows:
1. 100% methylmethacrylate (MMA), 2 days, store at

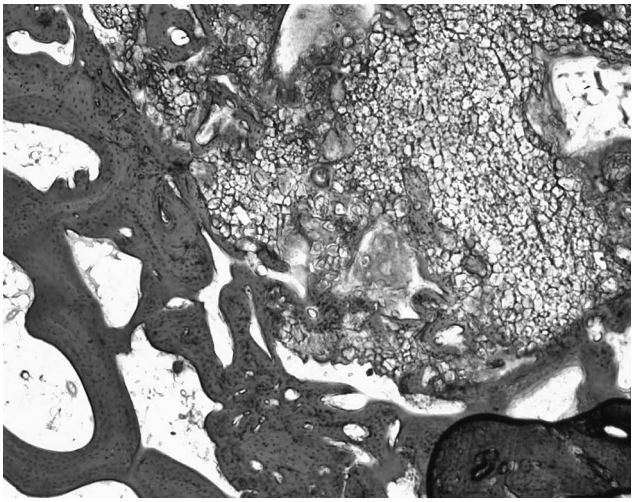

Figure 1. Trichrome-stained cross-sections of bone with neutralized glass ceramics four weeks after implantation. Overview in 25 – fold magnification, showing initial bone trabeculae in dark red, soft tissue in pink and remnants of the glass ceramics in turquoise.

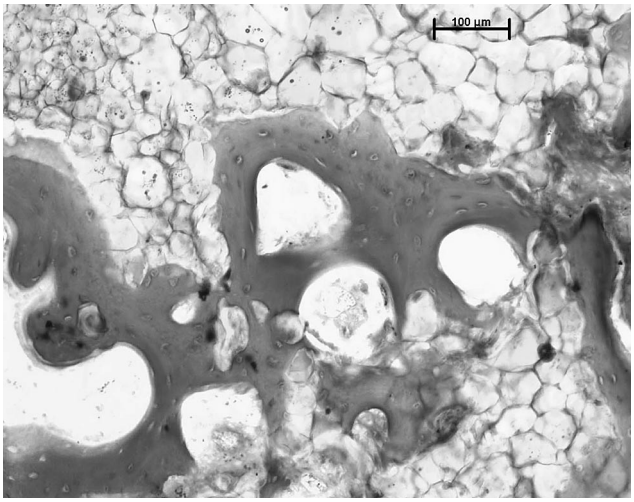

Figure 2. 100-fold magnification with nuclei appearing in dark blue and the osteiod border in blood red.

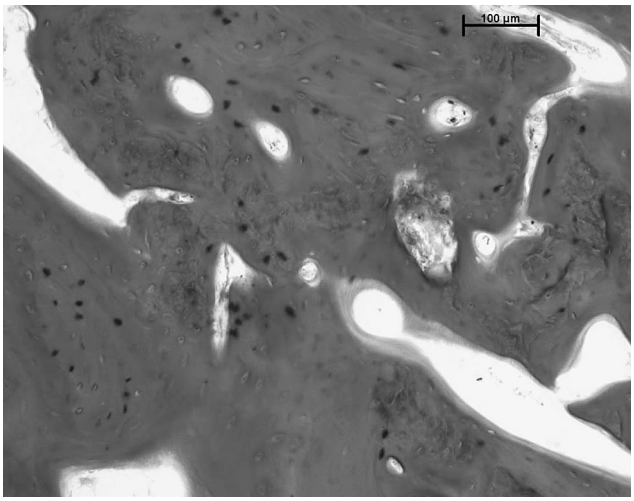

Figure 3. 100-fold magnification showing the close interface between new bone and ceramic substitute.

4° C (during this two days the specimens were placed 3 times for 20 minutes in a vacuum desiccator), change the MMA each day.
2. 100% MMA, 3 days, store at 4° C.
3. MMA and 2% dried benzoyl peroxide, 5 days, store at 4° C, change the MMA two time, during this five days the specimens were placed 3 times for 20 minutes in a vacuum desiccator.
4. MMA + 3,5 % dried benzoyl peroxide + 25 % dibutyl phthalate, store at 4° C, 7 days, no change of the MMA.
5. Store the spicemens at room temperature until totally polymerisation, appr. 14 days.

Sectioning

The embedded blocks were released from the glass vials and undecalcified sections of 100 um were sawed and grinded using an EXACT diamond saw system (Grünewald GmbH, Laudenbach. Germany).

Staining

Solutions

1. Weigert's iron haematoxylin solution (6).
2. Gio's trichrome solution (0,5 % phosphomolybdic acid,1 % orange g, 1 % light green, 2 % acid fuchsin, 1 % ponceau xylidine; solved in 1% acetic acid).

Staining procedure

1. Take sections for 15 sec in 0.5% phosphotungstic acid.
2. Place it at once in distilled water for 2 minutes.
3. Stain nuclei with Weigert's iron hematoxylin for 15 minutes.
4. Rinse in distilled water.
5. Wash in running water for 10 minutes.
6. Rinse in distilled water.
7. Stain in Gio's solution for 2–3 minutes under sight.
8. Rinse until clear in 1% acetic acid – 2 changes
9. 100% isopropanol – 3 changes.
10. Place in xylene.
11. Mount sections in a xylene soluble mounting medium.

RESULTS

Special colour effects of the new staining technique for the identification of remaining

synthetic bone substitutes are presented in the figures 1–3.

Initial bone trabeculae appear in dark red, soft tissue in pink and remnants of the glass ceramics in turquoise. Nuclei appear in dark blue and the osteiod border in blood red.

Colour effects of the new staining technique are summarized below:

Nuclei:	dark blue
Bone matrix:	dark red
Osteoid border:	blood red
Connective tissue:	pink
Synthetic bone substitute:	bright green to turquoise

DISCUSSION

This new staining method is fast to perform and reliable in differentiating remnants of synthetic bone substitutes from all other structures within the section. The appearance in the microscope is different to that after staining according to the Masson Goldner technique (4), which is the common method, at least in our country to visualize bone in connection to bone substitutes. The Masson Goldner technique staines old bone trabecula, new bone formation and remnants of synthetic bone substitutes in more or less the same colour (=green) (4), which makes the quantification of degradation rates of synthetic bone substitutes difficult.

CONCLUSION

The new staining technique is recommended as a routine method both for experimental and diagnostic purposes, it has more or less replaced the Masson-Goldner technique in our research labs, when synthetic bone substitutes are investigated.

ZÁVĚR

Autoři popisují podrobný postup při zalévání velkých vzorků kosti do metyl metakrylátu a novou metodu používanou k obarvení tenkých řezů nedekalcifikované kosti se syntetickými implantáty. Bylo dosaženo různých barvících efektů, které významně usnadňují hodnocení řezů kosti, nově se vytvářející kosti a zejména zbytků syntetických implantátů. Identifikace a kvantifikace zbytků implantátů je při použití běžných barvících postupů obtížná.

Autoři navrhují podrobný postup pro zalévání materiálu a barvení řezů a jejich montování.

References

1. DELLING, G.: Simplified metacrylate embedding method for undecalcified bone sections. Beitr. Pathol., 145: 100–5, 1972.
2. LACZKO, J. LEVAI, G.: A simple Differential Staining Method for Semi-thin Sections of ossifying Cartilage and Bone Tissues Embedded in Epoxy Resin. Mikroskopie, 31: 1–4, 1975.
3. MASSON, P.: Trichrome stainings and their preliminary technique. J. Tech. Meth., 12: 75–90, 1929.
4. ROMEIS, B.: Mikroskopische Technik, 17. Aufl., München, Wien, Baltimore, Urban u. Schwarzenberg 1989.
5. SCHENK, R.: Zur Histologischen Verarbeitung von unentkalkten Knochen. Acta anat., 60: 3–9, 1965.
6. WEIGERT, C.: Eine kleine Verbesserung der Hämatoxylin-van Gieson-Methode. Zwis Mikr., 21: 1–5, 1904.

Dr. S. Kessler,
Orthopedic Department, Sindelfingen District Hospital,
Arthur-Gruber Strasse 70,
71065 Sindelfingen
Germany
Phone: +49 - 7031 - 982481
Fax: +49 - 7031 - 982493
E-mail: s.kessler@kh-sindelfingen.de

Práce byla přijata 7. 4. 2008.