Technical Report
Scintigraphic Evaluation of Bone Formation in Göttingen Minipigs

by ***Schwarz ML[1,*], Seidling R[1], Mauermann E[1], Werner A[2], Steil V[3], Forsch E[4], Obertacke U[1], Becker K[5], Lehmann L[1]***

[1]Orthopaedic and Trauma Centre, Laboratory for Biomechanics and Experimental Orthopaedics, University Medical Centre Mannheim, University of Heidelberg, Germany

[2]Institute of Clinical Radiology and Nuclear Medicine, University Medical Centre Mannheim, University of Heidelberg, Germany

[3]Department of Radiation Oncology, University Medical Centre Mannheim, University of Heidelberg, Mannheim, Germany

[4]Department of Anesthesiology and Intensive Care Medicine, Medical Faculty Mannheim, University of Heidelberg, Germany

[5]IBF, University of Heidelberg, Germany

Summary

In experiments and processes requiring the application of nuclear tracers in large animals, statutory provisions and safety standards as well as a variety of techniques have to be regarded and employed.

In order to sufficiently analyze questions pertaining to osseointegration as well as the possibility of ectopic bone formation in Göttingen minipigs, we decided to use scintigraphic examinations using 99mTc-HDP (Technetium - hydroxymethane diphosphonate). In this study, metallic implants coated in different forms with rhBMP-2 (recombinant human bone morphogenetic protein-2) were surgically introduced into the pigs' femora. A total of 26 adult female minipigs (Ellegard, Dalmose, Denmark) averaging 40 months in age were post-surgically evaluated through the application of a radionuclide and its subsequent distribution using a scintillation camera. Each animal received approximately 10 MBq/kg BW (mega Becquerel per kilogram bodyweight).

This paper describes the procedures of anaesthesia, the quite challenging transvaginal- urethral catheterization, the application of a catheter in the jugular vein, the radionuclide injection and the disposal of the sacrificed animals under statutory provisions and safety standards.

The technical report reveals that the scintigraphic evaluation in large animal experiments is a practicable – yet sophisticated – method of examination and also strives to encourage further research groups to implement this elegant procedure.

Introduction

The application of nuclear tracers for the analysis of bone turnover in large animals requires several considerations pertaining to both safety and the techniques employed. Within a study dealing with the osseointegration of metallic (titanium) implants, we examined the possibility of ectopic bone formation resulting from the use of implants coated in rhBMP-2.

In this study, different forms of rhBMP-2 application were examined (*Jennissen 2002*; *Becker et al.*

**Correspondence:* Markus L. R. Schwarz, MD
Laboratory for Biomechanics and Experimental Orthopaedics, Orthopaedic and Trauma Centre
University Medical Centre Mannheim Theodor Kutzer Ufer 1 – 3, 68167 Mannheim, Germany
Tel +49 621 3834569
Fax +49 621 3834549
E-mail markus.schwarz@ortho.ma.uni-heidelberg.de

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2006) since some recent studies indicate a subsequent induction of ectopic bone synthesis as a result of rhBMP-2 use. A total of four implants were placed in each of the 26 animals, two per femur, one intercondylar and one intertrochanteric.

The use of 99mTc-HDP i.v. promises to be the most appropriate technique for detecting bone formation at any given region of the pig's body (*Banovac 2000; Varady et al. 2002*). In this study 26 female minipigs (Ellegard, Dalmose, Denmark) with an age of arund 40 months (median; max. 54, min. 24) were evaluated post operatively using this marker and a scintillation camera. The following text focuses on both legal and safety requirements as well as handling techniques of large animals in scintigraphic evaluation.

Legal requirements

Nuclear tracers must be injected in declared and legalized rooms according to the Radiation Protection Ordinance (*Deutsche Strahlenschutzverordnung 2001, 2002*). As a result, the pigs had to be transported from the animal laboratory to the Department of Nuclear Medicine in the University Hospital, Mannheim. The procedure was officially sanctioned by the Regierungspräsidium Karlsruhe, and encompassed the treatment of animals with nuclear markers including all relevant procedures as well as their housing within the facilities of a general research laboratory in the University Hospital, Mannheim (No: AZ: 35-9185.81/G-27/5 with extension).

All participating staff was required to take appropriate steps for radioactivity protection and monitoring, including the use of film-dosimeters and ring-dosimeters which were examined on a monthly basis.

Premedication, general anaesthesia and intubation

The animals were pre-medicated in the laboratory by means of an intramuscular injection consisting of 600 mg Ketamin (Ketaminhydrochlorid 10%, bela-Pharm GmbH & Co.KG, Vechta, Germany) and 45 mg Dormicum® (Midazolamhydrochlorid 15mg/3ml, Roche , Basle, Switzerland). The preparation room was equipped with an electrocardiogram (Lohmeier M211-371 (München, Germany) and a respirator (Narkomat, Heyer Anesthesia, Bad Ems, Germany) in stand-by. As the correct standardized use of a radionuclide requires strict intravenous application, up to five venous indwelling cannula (G14-G22, Vasofix® Braunüle®, B. Braun, Melsungen, Germany) were placed in the ear veins and fixated with two sutures each, thereby ensuring sufficient access to the venous system throughout the entire procedure. The cannulas were blocked with the appropriate mandrins (Mandrin for Vasofix®, B. Braun, Melsungen, Germany). The pigs were then anaesthetized with 40mg of Propofol® (2%, MCT Fresenius, Bad Homburg, Germany) i.v. Once the corneal reflex could no longer be evoked, the pigs were intubated (Rüsch Mikrolaryngeal-tubus, I.D.: 5.0 mm). The depth of anaesthesia was moniotored by heart rate and corneal reflex. An insufficient level of anaesthesia led to the application of 5mg/kg/BW Narcoren® (Pentobarbital-Natrium, Merial GmbH, Hallbergmoos, Germany) through a three way valve using 0.9 %NaCl (Delta Select GmbH, Heidelberg, Germany). In the rare case of excessive sedation, breath assistance was employed by the bag valve until spontaneous breathing resumed.

Transvaginal-urethral catheterization

Human in-patients are not required to be isolated following the injection of 99mTc. Furthermore, it is not necessary that their urine be disposed of using special procedures as long as the radioactivity is renally eliminated and diluted in the sewerage system. Nonetheless, the pigs' radioactive urine had to be collected by means of catheters in order to prevent radioactivity from contaminating the laboratory and staff. This was all the more necessary as the animals received two litres of saline solution over the course of two hours in order to ensure adequate circulation and dispersion of nuclides in the body and especially the bones. The pigs remained catheterised beyond their sacrifice, which occurred just prior to the scintigraphic imaging.

Consequently, safety precautions necessary for live animals were not applicable at this point, but radioactivity considerations obviously did not cease.
Consequently, the transvaginal-urethral catheterization was unavoidable. Unfortunately, the catheterization of female animals requires a certain level experience as described by DeLillo and Hansen (*2008*) in the case of dogs.

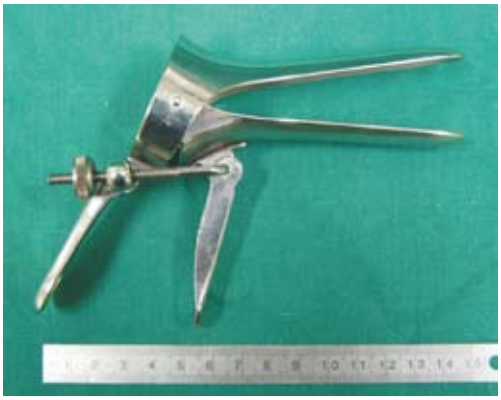


Figure 1. The speculum and its mechanics. The two branches were inserted vertically, thereby allowing access to the urethral orifice located on the ventral side of the vagina.

The transvaginal-urethral catheterization was carried out in an abdominal position with the hind legs stabilizing the pig. A gynaecological speculum (Fig. 1) was inserted into the vagina using Vaseline, a lubricant used for the urethral catheterization in men (Endosgel® steril, Farco-Pharma GmbH, Köln, Germany) as well as xylocaine spray (Xylocain Spray 2%, AstraZenca GmbH, Wedel, Germany) (Fig. 2). The lubricant and xylocaine were used to reduce friction and to prevent the vaginal mucosa from swelling, particularly in the urethral orifice. The speculum was inserted in a vertical fashion, thereby allowing unimpeded access to the urethral orifice, which is located in the ventral portion of the vagina (*DeLillo D & B Hansen, 2008)*. The required degree of openness of the speculum was determined, then speculum branches were fixated in the appropriate position and the xylocain-application nozzle was used to locate and infiltrate the small aperture of the urethra (Fig. 2). The end of the nozzle has the shape of a small olive, thereby both ideally locating the urethra by means of gentle forward pressure as well as simultaneous spraying and anaesthesizing the orifice and the urethra. The nozzle then remained in the urethra as a guide rail for positioning the catheter.

Figure 2. The nozzle (top) of the xylocaine spray (bottom) proved to be a useful instrument for probing the orifice and the urethra itself on account of its tip. During gentle insertion, xylocaine was applied, thereby lubricating, expanding and anaesthesizing the urethra. Once the nozzle was fully inserted, it was disconnected from the bottle and served as a guide for the catheter.

Standard sterile urinary catheters (14 Ch - 18 Ch; Folatex® Mentor, Porgès S.A.S., Le Plessis Robinson Cedex, France) were used for the catheterisation. On account of the orifice's location well within the vagina, the narrow anatomical structure and the soft material of the catheter itself, an inner stabilisation of the catheter was necessary (Fig. 3). Since the catheter was to transport and collect radioactive urine, a catheter with a large diameter was required, which made catheterisation even more difficult. Only the use of a mandrin of a central venous catheter (Cavafix®, B. Braun, Melsungen, Germany) (Fig. 3) and two self made spring steel wires (ap-

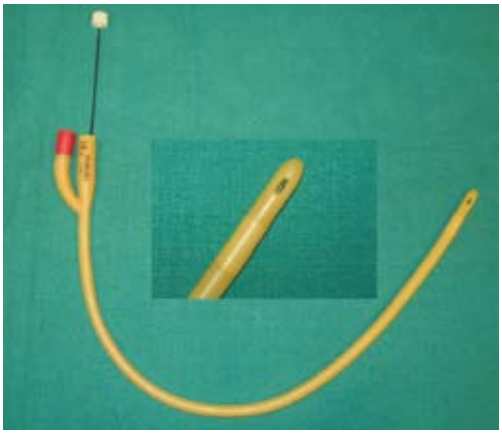


Figure 3. The urinary catheter armed with the mandrin of a central venous catheter. Detail: the mandrin can be seen in the apertures of the catheter's tip. For the catheterization process itself, the mandrin was fully inserted into the catheter, thereby stiffening the catheter.

prox. 100cm long and 0.8 and 1.2 mm in diameter) made the catheterisation possible.

For actual catheterisation, another bolus of 40 mg of Propofol® was injected in many cases, thereby reducing muscle / sphincter tension. Next, overholt – forceps were used to first grasp the stiffened catheter (catheter and wire/mandrin) and then gently push it along the guide rail, through the urethra and into the bladder, all the while generously applying lubricant. After the efflux of urine, which served as the proof of the correct catheter's position, the catheter was pushed forward until 3 cm protruded, blocked with 7 ml of saline, and then attached to the plastic urinary collection unit. A gentle tug on the catheter served as a test to ensure it remained in position.

Some practical comments to the catheterization procedure

The most successful way of inserting the catheter employed the use of the xylocaine spray's nozzle as rail guide. Following its insertion, the nozzle must be fixed by a second pair of forceps held off to the side. The stiffening of the catheter with the mandrin of a central venous catheter (Cavafix® see above) was mostly used and quite successful (Fig. 3).

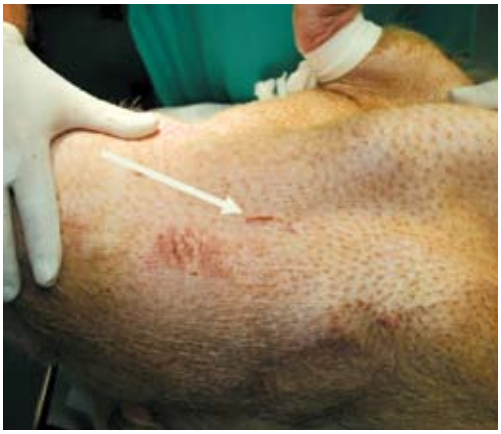
For probing both the orifice and the urethra a guiding wire (0.97mm diameter) provided in a catheterisation set (Easy Glide 8FR, Smith Medical Deutschland GmbH, Kirchseeon, Germany) was helpful in some cases.

Appropriate illumination is of the utmost importance. The operation lights were placed behind and just to the side of the head of the individual inserting the catheter. Nonetheless, additional head lamps may be helpful. In some cases, the successful catheterization was facilitated by turning the animal onto its right side and standing on the left near the animal's legs. The correct position of the catheter can only be assumed once urinary efflux has been observed, even if the catheter was easily inserted. Keep in mind that before efflux of urine can be observed, the mandrin stiffening the catheter has to be removed as it is placed in the lumen of the catheter.

Allow for sufficient time to insert the catheter, as this requires both patience and experience. The catheterisation took up to one hour in some cases! Obviously, the longer the process, the more propofol was required, but one must be aware of possible breath depression at the moment of injection.

Catheter in the jugular vein

After nuclide injection several litres of saline had to be infused in order to disperse the nuclides in the body, thereby achieving sufficient bone turnover. Initially, we had the intention of placing as many indwelling cannulas in the ear veins as possible (occasionally also the veins of the fore or hind limbs). However, some of the cannula ceased to function over time through clotting, transportation, etc. Therefore, we opted for the use of a central vein catheter providing a wide lumen. A flexible tube (Pumpleitung – DIN 58362 – SL – P Bioline, biocon, Mönchberg, Germany) with a calibre of approx.1.5 mm was placed in the jugular vein. This solution enabled us to draw blood when needed (up to 10 ml) and



Fugure 4. The skin incision (arrow) beneath the larynx at the right hand side. The anaesthesized animal is fixated in the supine position.

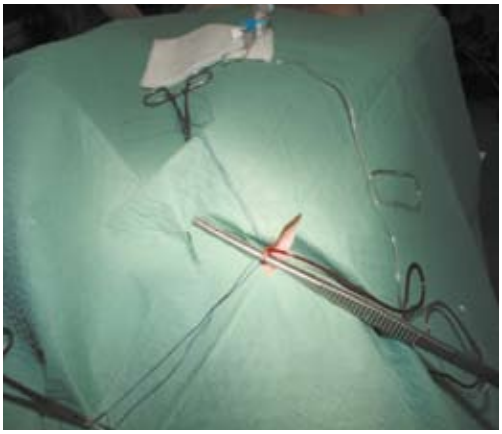


Figure 5. The jugular vein is carefully lifted out of the body and held by tweezers. The tube is inserted into the lumen as shown by its filling of blood. The ligature at the bottom of the figure is tightened in the apical direction while the ligature in direction of the heart is ready to be tightened as demonstrated by the prepared knot.

infuse great amounts of fluid in a quick and safe manner.

In the supine position, a cut with a length of approx. 4 cm was made 3-4 cm parallel to the median line beneath the laryngeal region (Fig. 4). Using long dissection scissors, the jugular vein was identified. The vein was gently lifted with a lid - retractor and held by tweezers above the skin's level (Fig. 5), thereby making the vessel easily accessible for manipulations. Next, the lumen was occluded through a ligature at the cranial end (POLYSORB®, tyco Healthcare Group LP, Norwalk, USA), so that the vessel drained. Following a small incision into the jugular vein, the catheter was inserted approximately 8 cm towards the heart. The correct position was determined by blood filling the tube which was then fixated in the vein by means of two ligatures. The skin was closed with monofil thread, covered with compresses, and fixed with a broad tape (Fixomull®, BSN Medical GmbH, Hamburg, Germany). The catheter was connected via a three-way valve with an administration set (R 87 RLS Luer-Lock IG-P Becton Dickinson GmbH & Co. KG Heidelberg, Germany) and saline (0.9 % NaCl, Delta Select, Pfullingen, Germany) was infused continuously.

Nuclide-injection, sacrificing and disposal

The injection of the 99mTc-HDP (hydroxymethane diphosphonate) radionuclide was performed in the Institute of Nuclear Medicine in the University Hospital, Mannheim. After examining the status of the venous cannulas in the ear, the nuclide was injected according to the animal's weight (approx. 10 MBq/kg BW; mean animal weight 56 kg; max. 77kg, min 35kg). As an alternative to the ear cannula, the jugular vein catheter could be used for application. However, the use of peripheral catheter/cannula is recommended in order to minimize the overlay of artefacts of bone structures with the contaminated catheter lumen. Furthermore, this enables one to precisely and easily identify the site of injection. After the injection, the cannula was rinsed thoroughly with 20ml saline (0.9 % NaCl, B. Braun Melsungen, Germany) and again bocked with the fitting mandrin.

Two litres of saline (see above) were infused and exactly two hours after the injection, the pig was sacrificed. Once a flat line in the ECG was identified,

the urine collector was exchanged and the pig was again transported to the Department of Nuclear Medicine for the post mortem scintillation images.
Following the examination, the body of the animal, the urinary catheters and all other potentially contaminated equipment was placed in a nuclear storage bunker with a temperature of -20°C. It was necessary to freeze the corpses to allow for ample time for radioactive decay (at least 36 hours). The time period was calculated as the six-fold half life of the used nuclide 99mTc. Following this period of time, the remains and waste were disposed of in the regular waste for research animals.

Conclusion

Within the framework of a study examining ectopic bone formation following the implantation of rhBMP-2 coated titanium implants, scintigraphic examinations were determined to be the most appropriate and refined procedure despite the challenges inherently involved in applying this method to large laboratory animals. The procedure may be also useful in examining further objectives, e.g. the examination of inflammatory processes. Considering current issues and development in the wide field of radionuclide imaging, its application in large animals may become more interesting for many researchers.
We were able to show that this ambitious procedure is operationally feasible, both on a technical level and also in terms of statutory provisions and safety standards. Finally, several experts must be involved in the examination procedure. In summary, the implementation of scintigraphic evaluation in large animal experiments is a practicable yet sophisticated method.

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