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Microcomputed tomographic, biomechanical and histological analyses of lumbar interbody fusion with iliac crest bone graft in a pig model

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Abstract

The main goal of this study was to assess the progress of vertebral stability after lumbar interbody fusion related to microcomputed tomography (micro CT), biomechanical analysis, and histological assessment towards spine fusion. Twelve male pigs were used; each underwent L2-3 discectomy and implantation of an iliac crest bone graft in two groups; six spines were harvested eight weeks (A1) and six spines 16 weeks (A2) after surgery (7 native spines for biomechanical analysis). The CT was performed by GE phoenix datos|x 2.0 with a sample drift correction. The samples were divided according to fusion quality. Biomechanical evaluation was carried out on the MTS Mini Bionix testing system. In the nondestructive mode, three cycles of pure bending moments were applied (5 Nm load limit) at a rate of 20 °/min in flexion (+40 °) and extension (-40 °). Two representative histological sections from four samples were obtained (A1, n = 2; A2, n = 2); areas of mature bone were quantified. In micro CT, better results were achieved in group A2 (not significant). Eight weeks after the operation, flexural stiffness decreased to 48% of its initial value for native cadavers ($P < 0.05$); after 16 weeks it was comparable to native cadavers, demonstrating the suitability of the implanted graft ($P < 0.05$). The newly formed bone tissue occupied an average area of 94.205 mm^2 (A1) and 26.240 mm^2 (A2). It was confirmed that micro CT, biomechanical analysis, and histological assessment are technically feasible and suitable for the evaluation of results of other methods of large bone defect treatment.

Bone replacement, spine, injury

The use of lumbar fusion procedures has rapidly increased in the USA and Europe over the last decade (Deyo et al. 2004; Resnick 2007). A large number of these procedures involve the use of bone grafts (Boden 2002). The most frequent indications for lumbar fusion involve degenerative spinal diseases leading to chronic pain, comminuted fractures of the vertebral body, congenital spine malformations, and bone defects after tumour resection. Despite the technical progress of spinal surgery and operative materials, the risk of vertebral fusion failure occurs in 5–35% of cases (Boden 1998). Successful fusion depends on a number of surgical and host factors including the selection of a bone graft or bone substitute with adequate osteoconductive, osteoinductive and osteogenic properties (Aebi 2007). Autografting has been considered the gold standard for bone graft

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procedures as it poses little risk of infection and rejection and is naturally osteoconductive, osteoinductive, and osteogenic. However, harvesting from the iliac crest can be associated with short and long-term morbidity in up to 22% of cases (Tan et al. 2007), as well as deformity, scarring and other surgical risks. To our best knowledge, no literature is available about the progress of intervertebral body fusion with a bone graft in a large animal model related to vertebral segmental stability. These facts should be helpful in the assessment of postoperative vertebral loading and could be used as a referential model for the testing of new inter-body implants.

The main goal of this experimental study was to assess the progress of vertebral segmental stability after lumbar interbody fusion (LIF) related to microcomputed tomography (micro CT), biomechanical analyses, and histological assessment towards spine fusion.

Materials and Methods

Animal model and study design

A total of 12 male pigs, 2.8 ± 0.2 months old, weighing 41 ± 9 kg, were used in this study. Each underwent lateral LIF with implantation of an iliac crest bone graft. The animals were divided into two groups of six; from each group, six spines were harvested 8 weeks (A1) and six spines 16 weeks (A2) after surgery. Seven cadaveric intact lumbar spines underwent biomechanical testing as control. All animal surgeries and experiments were performed in compliance with the Czech law on animal experiments and approved by the Czech Ministry of Agriculture. Pigs were housed individually with free access to water. General and neurological examination took place daily during the first week and twice a week later on. The pigs of both groups were euthanized by i.v. administration of embutramide, mebezonium iodide, tetracaine hydrochloride injectable solution at 6 ml/50 kg (T61, MSD Animal Health, Canada). A spine interval from T14 to L6 was excised and cleaned from surrounding tissues while preserving ligamentous stabilizers. Metal material was removed. No unusual reaction or any signs of releasing were observed around the implant.

Surgical method

Animals were premedicated and anaesthetized intramuscularly with tiletamine 2 mg/kg + zolazepam 2 mg/kg (Zoletil, Virbac, Czech Republic), ketamine 2 mg/kg (Narketan, Vetoquinol, France), and xylazine 2 mg/kg (Sedazine, Fort Dodge, USA). Intravenous anaesthesia was maintained by propofol infusion at 10 ml/h (Propofol 1%, Fresenius Kabi, Germany). Amoxicillin/clavulanic acid 15 mg/kg (Amoksiklav, Lek Pharmaceuticals, Slovenia) as prophylactic antibiotic treatment was administered once before operation.

After fluoroscopic identification, a left minilumbotomy incision and extraperitoneal approach to L2-3 space was performed. With fluoroscopy control, L2-3 discectomy and implantation of the iliac crest bone graft was done. The operated segment was stabilized with titanium monoaxial screws, rods, and plates (U-Smart anterior system, Bone care, USA). After surgery, the pigs were monitored until full recovery from anaesthesia. A prophylactic dose of ATB was administered (alamycin LA 300 inj, 30 mg/kg, i.m.). Pigs received ketoprofenum analgesics 0.3 ml/10 kg i.m. (Comforion Vet, Orion Pharma Animal Health, Finland) regularly for three days after the surgical procedure.

Micro CT evaluation

For the purposes of CT measurement, the pig spine was sealed in plastic foil to eliminate the movement and drying of the sample. To further minimize the sample movement, it was placed in a paper tube. The prepared sample was placed onto the CT table so that the position of the measured LIF area was clear and the two vertebrae with the bone graft were exactly in the middle of the field of view.

The micro CT system GE phoenix v|tome|x L 240 (GE Sensing & Inspection Technologies GmbH, Germany) equipped with a nanofocus180 kV/15 W X-ray tube and flat panel detector DXR 250 was used for the tomographic measurement. The X-ray tube was set at an accelerating voltage of 100 kV and a current of 300 µA. The X-ray spectrum was filtered by a 1.5 mm thick aluminium filter. The detector exposure time was 400 ms in every 2200 positions. The measurement was performed at the constant temperature of 21 °C. The voxel size was 40 µm.

The tomographic reconstruction was performed by GE phoenix datos|x 2.0 with a sample drift correction, beam hardening correction and noise filtration. VG Studio MAX 3.1 (Volume Graphics GmbH, Germany) conducted the registration of the object according to the top - cranialis and bottom - caudalis (ventralis, dorsalis) of the sample. The registration simplifies the analysis of LIF. The bone graft was implemented always in the same position and the LIF quality was analysed.

The samples were divided into the four groups of different fusion qualities according to CT appearance; examples are shown in Fig. 1 (Plate IV):

- Grade I (complete fusion) implies cortical union of the bone graft at cranial and caudal ends of the bone and continuity of the vertebral trabecular pattern between the bone graft and the adjacent cranial and caudal vertebral bodies.

- Grade II (partial fusion) implies cortical union of the bone graft at least at one of the ends and also with partial or absent trabecular continuity between the medullary bone graft and the adjacent vertebral body at one or both ends.
- Grade III (unipolar pseudarthrosis) denotes cranial or caudal cortical non-union of the bone graft with associated central trabecular discontinuity. The adjacent vertebral bodies are connected along the body but without any interbody fusion with the bone graft.
- Grade IV (bipolar pseudarthrosis) suggests both superior and inferior cortical non-union with a complete lack of central trabecular continuity. Complete absorption of the bone graft was observed in several cases.

The measured results were objectively statistically evaluated by non-parametric Chi square test; significance was accepted at $P \leq 0.05$.

Biomechanical testing

Biomechanical evaluation of lumbar spinal stability after surgical intervertebral fusion was carried out on the MTS Mini Bionix testing system (MTS, USA). Flexural stiffness in the extension of the lumbar spine cadavers (T15-L6) free of all soft tissues except for intervertebral ligaments and facet joint capsules was evaluated as native specimens (N, n = 7) and experimental specimens of the two groups (A1, n = 4; A2, n = 4). Cadavers were anchored to the Bionix Spine Kinematics System platform (Bionix 370.02, MTS, USA). Pure bending moment was applied to rigidly mount the inferior end (L6). The superior end (T15) was unconstrained, free to move in the vertical direction and to rotate. In the nondestructive mode, 3 cycles of pure bending moments were applied (5 Nm load limit) at a rate of 20 °/min in flexion (+40 °) and extension (-40 °). Before the start of a new cycle, the specimen was returned to its initial neutral position and unloaded. Based on the flexural moment (Nm) - angular displacement (degrees) curves, the flexural stiffness of specimens in extension was determined. The six-axe force-moment sensor (Mini 45 SI-580-20, MTS, U.S.A.) was used for load measurement (Plate V, Fig. 2). Statistical analysis was performed using statistical software (STATGRAPHICS Centurion XVII, StatPoint, USA). Non-parametric analysis was employed since either the assumption of normality or homoscedasticity were violated and, subsequently, Kruskal-Wallis test for multiple comparisons was used with subsequent pairwise comparisons between the average ranks of the three groups using the Bonferroni procedure. Significance was accepted at $P \leq 0.05$.

Histological evaluation

In order to conduct a scientific and accurate histomorphological analysis, two representative histological sections of the intervertebral segment in the sagittal plane were obtained from a total four samples from both groups (A1, n = 2; A2, n = 2). Evaluation and photographic documentation were performed on an Olympus BX45 microscope (Olympus Optical, Tokyo, Japan) (Plate VI, Fig. 3). Olympus Viewfinder Lite™ software was used to acquire and process images. The sections with the largest area of newly formed bony tissue were scanned with the ZEISS Axio Scan Z1 Digital Slide Scanner and the areas of mature bone were exactly quantified using the Imaging Software ZEN 2.6 (blue edition) software.

Results

Twelve pigs were operated during the experiment without perioperative complications. One pig from group A1 had to be euthanized on the 10th day for sudden paralysis of the hind legs. The reason was the formation of an abscess in the fused intervertebral space and a spine collapse with spinal nerve lesions. In order to maintain the number of animals, this pig was replaced by a new animal that was subjected to the same methodology without further complications.

Table 1. Samples of pig spines with corresponding obtained fusion grades.

Grade	A1 (n = 6)	A2 (n = 6)
I	3	1
II	1	3
III	1	2
IV	1	0

A1 – spines harvested 8 weeks after surgery; A2 – spines harvested 16 weeks after surgery

Micro CT evaluation

Six measurements were conducted for each pig in both groups after the sacrificing. The samples were classified into the previously mentioned groups and the results of the analysis are shown in the Table 1. Better results were achieved in group A2, however, no statistically significant difference was found between groups.

Biomechanical testing

The flexural stiffness of native cadavers (N) reached 1.39 (0.96–1.66) Nm/deg (median and interquartile range). Eight weeks after the application of the bone graft, the flexural stiffness of spine cadavers (A1) decreased to 48% of its initial value for native cadavers 0.73 (0.59–0.83) Nm/deg ($P < 0.05$). After further 8 weeks, the stiffness of the cadavers with the bone graft (A2) increased up to 45% and regained the values comparable to the native cadavers, i.e. 1.06 (0.90–1.12) Nm/deg ($P < 0.05$). The flexural stiffness of cadavers 16 weeks after implantation, comparable to native cadavers, demonstrates the suitability of the implanted graft (Fig. 4).

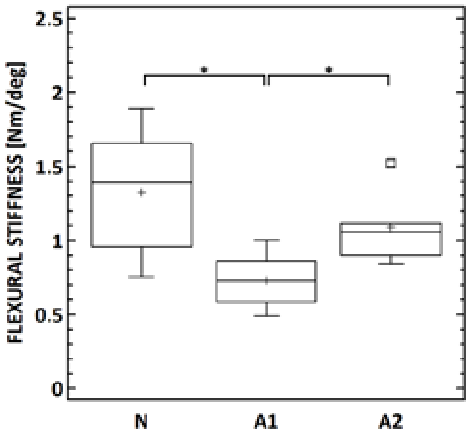

Fig. 4. Flexural stiffness of native lumbar spine cadavers (N), specimens 8 (A1) and 16 (A2) weeks after lumbotomy and autologous iliac crest bone graft insertion into the intervertebral L2-3 space; "*" denotes statistically significant differences (Kruskal-Wallis test with the Bonferroni procedure, 0.05)

Histological evaluation

The histomorphological analysis showed significant differences between both groups after 8 (A1) and 16 weeks (A2). The newly formed bone tissue in the defect occupied an average area of 94.205 mm^2 in group A1 and 26.240 mm^2 in group A2 in the standardized samples (Table 2). The bone formation in group A1 corresponds to the presence of the remaining bone graft; in group A2 we found a decrease of the bone formation area and a collapsed and partly resorbed bone graft.

Table 2. Bone tissue area calculation in representative samples.

Sample	Bone area in section 1 (μm^2)	Bone area in section 2 (μm^2)	Sum (μm^2)	Mean (μm^2)
A1 (Pig 10)	97 410 129	0	97 410 129	94 205 234
A1 (Pig 11)	44 449 951	46 550 388	91 000 339	
A2 (Pig 1)	2 917 584	1 488 668	4 406 252	26 240 220
A2 (Pig 4)	26 662 360	21 411 828	48 074 188	

A1 – spines harvested 8 weeks after surgery; A2 – spines harvested 16 weeks after surgery

In twelve pig models, we demonstrated the mechanical strength recovery of the spine after intervertebral fusion; however, it very likely does not depend on the degree of bone tissue healing and the amount of bone mass. The main goal of the work was to verify that the proposed methods of micro CT, biomechanical analyses, and histological assessment are technically feasible and objectively evaluable. At this point, it can be established that all three examinations could be used together for the evaluation of other methods of treatment of lumbar interbody fusion, for example using biomaterials, synthetic bones or new implants.

Discussion

The treatment of large bone defects is a major problem currently in the surgical (orthopaedics, traumatology) and internal medicine fields (oncology). Finding a suitable method or alternative filling is the goal of many research studies and grant projects (Nečas et al. 2010). Some of them involve orthopaedic (surgical) solutions (prolongation, spongioplasty, bone graft implantation) but more often clinical surgeons cooperate with bioengineers, looking for new bone replacements with suitable properties (Nečas et al. 2008). A few years ago, the use of stem cells seemed very promising. After these progenitors started to be used, they were coming from various sources and in many types, some of which unexplored, and complications started to occur, not only in regard to the technology and medical use but also in the issue of ethics. Research using stem cells (embryonic, mesenchymal) (Nečas et. al. 2008; Planka et al. 2012; Atesok et al. 2017) is still ongoing but the focus if now on non-cellular materials (Srnec et al. 2020). Such materials are less complicated to handle and safe to implant. Today's biotechnology allows to prepare an almost exact copy of bone mass (carrier) and then patiently search for ideal bioactive substances to stimulate bone proliferation and bone formation. However, all such experiments need a methodology to evaluate the clinical effect and healing of the defect to allow objective comparison of individual modalities. Therefore, we devoted the first part of our experiment to the study of possible modalities, the elaboration of methods of implementation and evaluation, and the verification of possible statistical evaluation of results.

The porcine model was chosen and the chosen method of treatment was spongioplasty with a bone graft excised from the hip. The porcine model is the most widely used experimental animal in the field of medical research in our conditions. There are well-developed methodologies for general anaesthesia including preoperative care, pigs tolerate the postoperative period well, and research facilities are adapted to the appropriate care (Crha et al. 2011; Linard et al. 2018). The lumbar spine was chosen, because the stability of the spine remains after the defect is created; in addition, the stability of the defect can be safely ensured by osteosynthetic material as verified by Veselý et al. (2008). If we were to select the femur, we would not be able to achieve sufficient rest for the healing, and rigid osteosynthesis would have to be used. Even so, we would probably observe numerous failures and a need for reoperation which would make the evaluation of the results very complicated with the relatively small number of experimental animals.

Micro CT examination was made possible thanks to cooperation with the Central European Institute of Technology (CEITEC). The methodology for evaluating vertebral body fusion was based on the work of Inamdar et al. (2006). We defined four stages of intervertebral fusion. The individual results could be compared statistically. The micro CT method was used to evaluate the results of their experiments e.g. by Xu et al. (2018), as well as Daentzer et al. (2014) who correlated those results with biomechanical evaluation, too. We consider micro CT to be a very detailed examination that cannot be replaced by simple X-ray. This should be used for perioperative verification of the location of the stabilizing implant and for the postoperative monitoring of healing, however, micro CT examination is required for the final evaluation of bone formation (Kroeze et al. 2015).

Biomechanical evaluation is necessary to verify the clinical effect of treatment. The main goal of the therapy is to obtain a mechanically strong healing of the bone defect. The examination was performed according to technological standards and the evaluation was similar to those used in the studies by Abbah et al. (2011) and Tang et al. (2015). Many standardized procedures had to be followed during this examination, the exact level of the spine that had to be dissected (T15-L6), the structures that had to be preserved (ligamentum longitudinale anterius and posterius, intervertebral discs, articular processes and joint capsule) and had to be resected (all muscle tissue, transverse processes and ribs, spinous processes at T15 and L6). They had to be fixed immediately and stored in a vacuum container, so that they were not degraded during transport to the biomechanical laboratory, especially when micro CT was still performed. Inconsistencies in these individual steps could be a possible source of failed results and should be avoided.

A limitation of the microscopic examination was that final histological specimens were created in only two animals from each group. However, it was possible to prove the technical feasibility and applicability of the method, because histological samples were made from each animal repeated many times. It was thus possible to verify the possibility and accuracy of measuring the area of newly formed bone by named microscopic software. The selection of the two representative samples corresponded to the sections with the highest bone density. Histological examination, unlike micro CT, confirmed a newly formed bone with cellular elements. Histological criteria for qualitative and quantitative assessment of bone formation are a difficult issue solved in many experiments. The method of comprehensive evaluation seems to be very objective. The primary condition remains that it is necessary to identify the mature bone mass, with all the components (non-cellular and cellular). In most similar works, only the presence of bone tissue is evaluated, often in detail by different types of staining (Rentsch et al. 2014), but the requirement to objectify the amount of new bone tissue is innovative in our view.

If we compare all three parameters, we find an interesting situation that obviously the strength of the spine in the operated level does not depend on the proven amount of bone mass. Although CT and microscopic examination show a smaller proportion of bone mass, biomechanical strength returns to the native spine over a longer time period. However, our main goal was not to look for a difference between experimental groups, but to verify the technical feasibility and objectivity of individual examination methods, which was clearly achieved. This set of evaluation methods can be used to compare the results of more methods of treatment of large bone defects.

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Fig. 1. Examples of samples sorted in to the groups according to the quality of fusion: A) no fusion observed (control specimen), B) fusion of the vertebrae is visible but it is not in the area of the bone graft (Grade IV), C) fusion in the area of the bone graft is presented but it is not completed (Grade II), D) fusion is clearly visible and entirely joins the vertebrae in the area of the bone graft (Grade I).

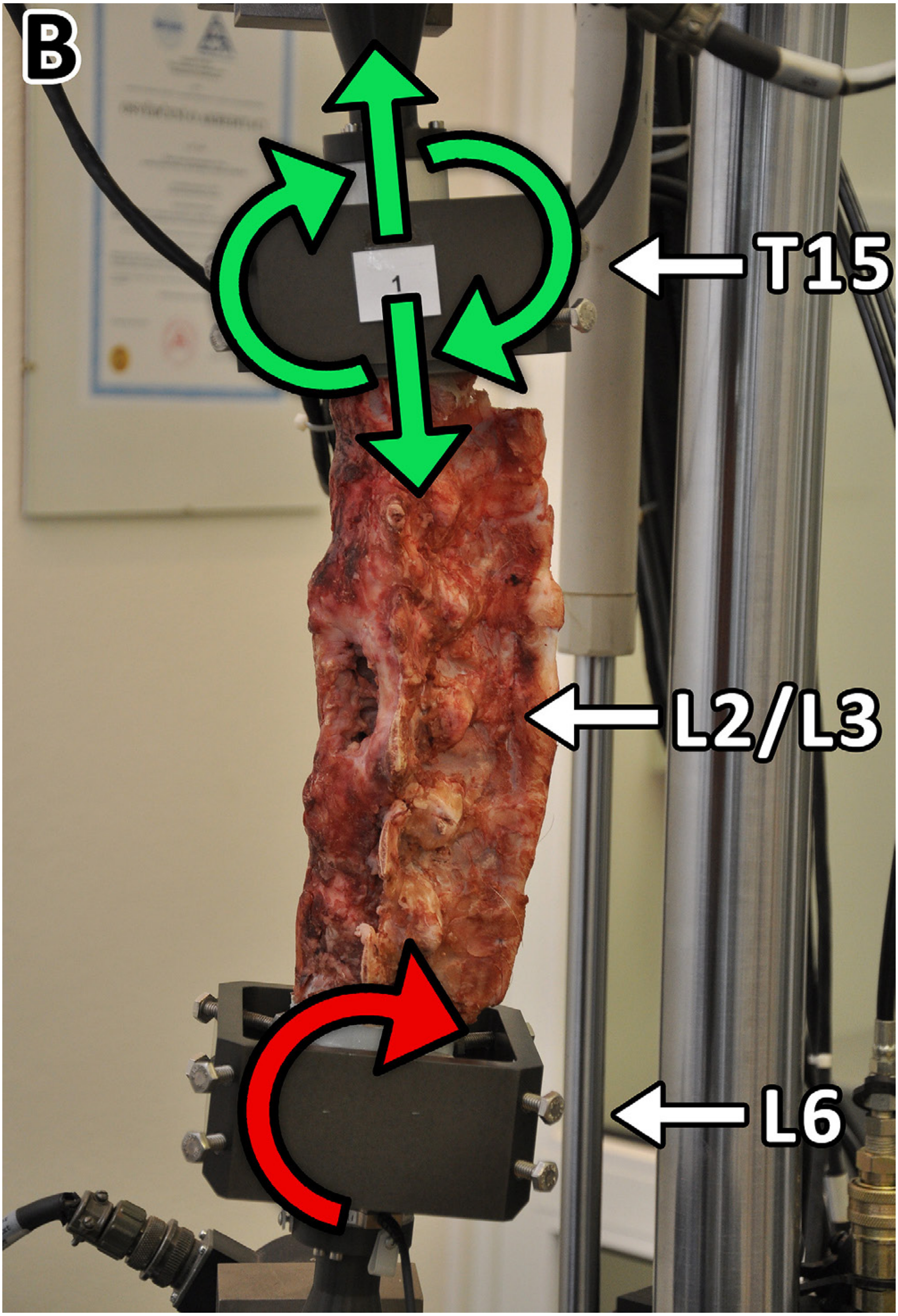

Fig. 2. The six-axes movement of the specimen by biomechanical testing

Plate VI

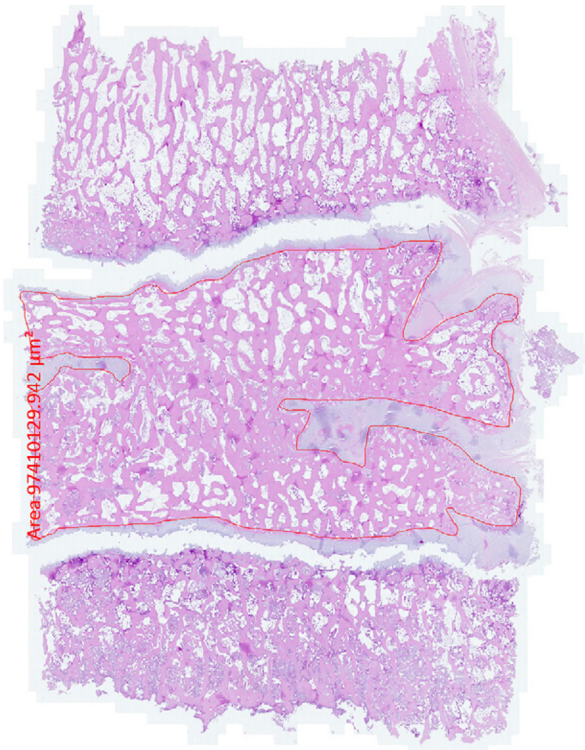

Fig. 3. Bone tissue area measurement in representative samples (HE, × 20 magnification, Pig 10, Group A1)