

Use of allogenic stem cells for the prevention of bone bridge formation in miniature pigs

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Short title:

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Summary

This study draws on an experiment which was previously carried out on the model of a New Zealand white rabbit. Allogenic Mesenchymal stem cells (MSCs) were transplanted into an iatrogenically-created defect in the lateral section of the distal physis of the left femur in 10 miniature pigs. The right femur with the same defect served as a control. To transfer MSCs, a freshly prepared porous scaffold was used, based on collagen and chitosan, constituting a compact tube into which MSCs were implanted. The pigs were euthanized 4 months after the transplantation. On average, the left femur with transplanted MSCs grew more in length ($0,56 \pm 0,14$ cm) compared with right femurs with physeal defect without transplanted MSCs ($0,14$ cm \pm 0.3 cm). The average angular (valgus) deformity of the left femur had an angle point of $0,78^\circ$, following measurement and X-ray examination, whereas in the right femur without transplantation it was $3,7^\circ$. The initial results indicate that preventive transplantation of MSCs into a physeal defect may prevent valgus deformity formation and probably also reduce disorders of the longitudinal bone growth. This part of our experiment is significant in its effort to advance clinical application in human medicine by using pig as a model, which is the next step after experimenting on rabbits.

Keywords: growth plate, injury, physis, limb deformity, MSCs

Introduction

The aim of our study was to verify the capacity of mesenchymal stem cells (MSCs) to repair a partial defect in the distal femoral physis on an experimental model of miniature pigs. A similar experiment preceded the study, using New Zealand white rabbit as a model with promising results (Plánka et al., 2007; Gál et al., 2007). In the majority of similarly-oriented studies, rabbit was chosen as an experimental model (Ahn et al., 2004; Chen et al., 2003), also providing encouraging results. Few cases of MSC transplantation into a physal defect in pigs have been described in literature so far. There are several works dealing with the positive effects of autologous chondrocyte transplants (Kawabe et al., 1987; Foster et al., 1990; Gál et al., 2002, Filova et al., 2007). This method is a modern modification of a widely used surgical procedure which includes physal bone bridge resection and filling the post-resection defect with an allogenic fat graft (Langeskiold et al., 1981), a fascia graft or a ligament graft (Janarv et al., 1998), possibly with silicone substitutes. Growth plate injury and results of therapy depended up age and type of epiphyseolysis .

Instead of a gel scaffold based on Tissucol which was previously applied (Plánka et al., 2007; Filova et al., 2007), we used a MSC transplant and a new scaffold in the form of a small porous cylinder consisting of 0,5% of collagen and 30% of chitosan (Jančář et al., 2007). The scaffold served as a compact and solid structure which we were able to implant into the created defect by means of the press and fit technique.

Our decision to use allogenic MSCs was based on their potential clinical application. MSCs could be transplanted straight into defects in emergency physal injuries and bone bridge formation could be prevented in this way. Bone bridge is an adverse complication of physal fractures in children. In dependence on the location (central, peripheral) of a bone bridge, the affected limb might become shorter or show angular deformity. Also, a combination of both these growth abnormalities might be observed (Campbell et al., 1959; Ford and Key, 1956;

Key and Ford, 1958). Limb deformity affects mostly medium and large breeds of dogs that are injured before 5 months of age (the most severe deformities develop after Salter-Harris injuries of distal physis of ulna) (Fox, 1984; Henney and Gambardella, 1989; Piermattei and Flo, 1990). It has been reported that 25-35% of physal fractures in humans lead to shortening or deformity of injured bone and that 10% of these fractures result in functional problems with affected limb (Mann and Rajmaira, 1990; Mizuta et al., 1987). Bone deformity is associated with 5-10% of physal closures in dogs. In most of these cases, it is necessary to perform corrective osteotomy to restore normal limb functions (Maretta and Schrader 1983). Prior to our experiment, we expected the femur which had a partial defect in the distal physis filled with a transplant (MSCs on a scaffold) to show no significant deformities during its future growth. The other femur served as a control. A separate control group of experimental animals was not established deliberately. By using the other femur in the same animal as a control, we were able to compare two different treatment methods in identical regenerative and healing conditions.

Methods

The experimental group consisted of 11 miniature pigs which were obtained from certified breeding at the Institute of Animal Physiology and Genetics of the Academy of Sciences of the Czech Republic. At the time of surgery, the age of animals ranged between 2,5 and 3 months and their weight did not vary significantly ($14,4 \pm 3,2$ kg).

Preparation of the stem cells

The bone marrow blood was aspirated from os illium (*ala osis illi*) into the 5 mL syringe with 2 mL Dulbecco's Phosphate Buffered Saline (PBS) with 2% Fetal Bovine Serum (FBS, StemCell Technologies) and 5 IU heparin/mL connected with a hypodermic needle (20G/40

mm). Under sterile conditions, the bone marrow blood (about 4 mL) was deposited over 3 mL of Ficoll-Paque PLUS (StemCell Technologies). After centrifugation at 400g for 30 min at room temperature, the dense gradient separated erythrocytes and granulocytes as a pellet in a bottom part of the tube; mononuclear cells were situated in an opalescent layer between Ficoll and blood plasma. This layer was taken out, washed in a culture medium (see below) and used for propagation under *in vitro* conditions. The average amount of mononuclear cells from each isolation was 20×10^6 cells. Cell number and viability was analyzed on Vi-CELL (Series Cell Viability Analyzers) and about 90% of viable cells were detected.

The cells were tested by means of the established positive markers for MSCs (CD29, CD44, CD90, CD105; CD147 has been recently added in several studies). The panel of antibodies used was: anti-CD29 (clone MEM-101A), anti-CD105 (clone MEM 229), anti-CD147 (clone MEM-M6/2, Exbio Praha a.s., Prague, Czech Republic), anti-CD44 (clone IM7), anti-CD90 (clone 5E10, BD Biosciences, San Jose, CA USA), anti-CD45 (clone K252 - 1E4, AbD Serotec, Kidlington, UK).

Culture, labeling and differentiation of mesenchymal stem cells

Cells were seeded in 75-cm² tissue culture plastic flasks at a density of approximately 5×10^5 cells/cm² and cultured at 37°C in humidified atmosphere with 5% of CO₂. The culture medium was α -MEM medium (Gibco) supplemented with 10% FBS (Sigma Aldrich) and gentamycin (50 mg/mL, Sigma Aldrich). After 24h of culture, the non-adherent cells were removed and during the subsequent culture (3 weeks) the medium was exchanged every third day. The first colonies of mesenchymal stem cells appeared after 4 to 5 days of culture and the 80% of confluence was achieved after 10 days of culture. Cells were passaged with trypsin 0.5% trypsin-EDTA solution (Sigma Aldrich) for 5 min at 37°C and replated in 150 cm² at a density of 5000-6000 cells/cm².

During the last three days of culture, cells were labeled with nano-particles of iron oxide (Resovist, 0.5 mmol Fe/mL, Schering). Resovist was added in concentration 1 μ L/mL culture medium. For labeling with a fluorescent dye CM-Dil (Molecular Probes) in concentration 5 μ g/2,5 mL PBS, cells were harvested on day of transplantation, incubated for 5 min at 37°C and for 15 min at 4°C. At the end of labeling cells were thoroughly washed in PBS. To induce chondrogenic differentiation (Miura et al. 2002), the labeled cells were given in the differentiation medium composed of α -MEM supplemented with 100 ng/mL recombinant human TGF β_1 (R&D Systems), 100 nM dexamethasone (Medochemie), 50 μ g/ml L-ascorbic acid 2-phosphate (Sigma Aldrich), 1% insulin-transferrin-selenium A (Gibco) for 30 min. Subsequently, the cells were centrifuged at 700g for 5 min, and cell pellets were prepared for their deposition in a scaffold.

Scaffold preparation

Initially, the scaffolds were prepared in the form of flat spongy semi-structures of the required thickness which were cut by surgical instruments to obtain matrices of the desired dimensions and these structures were then implanted with stem cells. Based on in-vitro evaluation performed at the Institute of Experimental Medicine of the Academy of Sciences of the Czech Republic, a collagen-based scaffold containing micro- and nanofibers made of chitosan was selected as the most suitable material which meets specific requirements (Koláčna et al., 2007): the required biological features, stability in simulated body environment, biomechanical response and the scaffold behaviour at the site of the planned implantation (ex vivo insertion of the scaffold into an iatrogenic defect in articular surface and tests of the mechanical properties of the scaffold on prepared knee joints of pigs in cooperation with the University of Veterinary and Pharmaceutical Sciences Brno and Masaryk University). The lyophilization process was optimized in order to obtain interconnected pores of approximately 100 μ m. With regard to successive testing of the suitable composition of a three-dimensional

carrier of MSCs, we can conclude that a small porous cylinder consisting of 0,5% of collagen and 30% of chitosan, cross-linked by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and catalysed by NHS (N-Hydroxysuccinimide 98%), with chitosan-nanofibers-based structure, seems to be an optimum choice. To facilitate manipulation during implantation, after lyophilization the cylinder-like substrates were submitted so that they could be implanted with cells in a 96-well plate.

Surgical procedures

All animals were intramuscularly administered tiletamine-zolazepam (Zoletil 100, Virbac, France) in 2 mg/kg dosing, xylazine (Sedazine, Fort Dodge, USA) in 2 mg/kg dosing and ketamine (Ketaset, Fort Dodge, USA) in 2 mg/kg dosing. All substances were administered together in a single syringe. After the start of sedation in 10 minutes after administration, intravenous catheter with propofol was inserted in the ear vein (Propofol 1%, Fresenius, Austria) in 1 mg/kg dose and endotracheal tube was inserted in pigs. All pigs were put into right lateral position and connected to the inhalation circle rebreathing system. They were supplied with a mixture of oxygen and air (1:1) in the amount of 25 ml/kg/min. All pigs were connected to a vital functions monitor (Datex Cardocap II, USA). The logged values included heart rate (HR), respiratory rate (RR), mean arterial pressure (MAP), hemoglobin saturation by oxygen (SpO₂) and end-tidal CO₂ concentration (ETCO₂). HR was measured using a 3-lead EKG with electrodes located on the patient's chest. MAP was measured using a disposable blood pressure transducer connected to a monitor after calibration. The pressure transducer was connected to the arterial access port (*a. auricularis* on contralateral ear than venous access) using extension tubing filled with heparinized saline (200 IU heparin ml⁻¹, Heparin, Léčiva, Czech Republic). RR and ETCO₂ were logged using side-stream with a sensor connected on the tip of endotracheal tube. SpO₂ was measured with a sensor connected to the patient's tongue.

After inducing anesthesia, hair was removed from the area of both knee joints of the miniature pig and the skin was washed with soap water and an iodine solution several times. In the final stage of aseptic precautions the operative field was exposed to chlorhexidine and the pig was positioned on the right hip.

Lateral arthrotomy was performed on the left knee joint (Shoja et al., 2002). After visualizing the lateral condyle of distal femur, a 6 mm screw was used to drill a canal across the growth plate (the Colibri battery-driven power tool; SYNTHES, USA). The diameter of the screw was selected with regard to our aim to destroy at least 9% of the growth zone surface (Janarv et al., 1998). The defect was drilled to a depth of 15 mm dorsolaterally from the insertion of *m. extensor digitorum longus*. The canal was drilled in dorsomedial direction in order to cause damage to lateral part of distal femoral physis including adjacent parts of epiphysis and metaphysis.

The scaffold with MSCs was inserted into a freshly created defect in the physis and its entry was sutured using the edges of periosteum where possible. The stifle joint was lavaged with Ringer lactat solution (Ringer Lactat I.V.Inf., Braun Medical AG). The joint capsule was closed with interrupted suture (PDS 1/0, Ethicon). Subcutaneous layer was closed with continuous suture using 2/0 PDS (PDS 2/0, Ethicon). The skin was closed with simple interrupted suture using 2/0 Ethilon (Ethilon, Ethicon). Afterwards, a defect in lateral part of distal epiphyseal plate of right femur was created similarly. However, the canal drilled in the condyle was not filled with implant (i.e. the scaffold with autogenous MSCs).

After the surgery, antagonization of all three anesthetic components was performed using a combination of naloxone (0.03 mg/kg (INTRENON inj., Léčiva a.s.)) + flumazenil (0.1 mg/kg (ANEXATE, Hoffmann-La Roche Ltd.)) + atipamezole (1,0 mg/kg (ANTISEDAN inj. ad us. vet., Pfizer Animal Health)) that was administered intramuscularly. Analgesia in the post-operative period was achieved by the application of carprofen (RIMADYL inj. ad us.

vet., Pfizer Animal Health) in the dose of 2 mg/kg/day for three days after the surgery. Following recovery from the surgery, the animals were allowed to walk freely and weight-bear as tolerated. During the whole study period, the animals were fed, handled and housed according to the principles of welfare (Sýkora et al., 1983). At the end of our experiment (4 months after the surgery), all animals were lege artis euthanized. First, they were put under general anesthesia using intravenous thiopental in the dose of 20 mg/kg. Then they were given intravenous T 61 inj. ad us. vet. (Hoechst Roussel Vet.) in the dose of 1 ml pro toto.

All procedures were carried out with consent of the Ethical Committee (No. 46613/2003-1020).

Bone length discrepancy and femoral valgus deformity measurements

Each pig was subjected to radiological examination on the day of transplantation and right after euthanasia. Bone length discrepancy and valgus deformity were measured from radiographs. Measurement of the length of left femur (with physeal defect and transplanted MSCs) and right femur (with physeal defect without transplanted MSCs) was done from radiographs of femur in craniocaudal (CC) projection. Actual length of femur and the angle of valgus deformity of distal femur were measured. The measurements were performed separately by three independent observers. The measured values were averaged to calculate the arithmetic mean.

Histological findings

Healing of the defect was investigated histologically using haematoxylin and eosin staining. Following the excision of femurs of the euthanized pigs, femoral distal epiphyses were placed in 10% solution of formalin and stabilized. They were then decalcified in Leavy's solution and gradually dehydrated in solutions with increasing concentration of alcohol to enable embedding them in paraffin. Ultrathin paraffin sections of distal femur 0.1 mm in thickness

were stained with haematoxylin and eosin (HE) and subjected to microscopy. The site of defect in the growth plate of femur was examined histologically.

To verify the formation of developed hyaline cartilage, we used PAS staining (detection of acid mucopolysaccharides found in the intercellular substance of hyaline cartilage) and immunohistochemical staining with an antibody against type-II collagen (type of collagen which is the basis for hyaline cartilage). The origin of chondrocytes located in the physeal defect was confirmed by Pearls method, i.e. the reaction of iron nanoparticles with Berlin blue, and by immunofluorescence with CM-DiI.

Statistical evaluation

Means and standard deviations were calculated for the length and valgus deformity of right femur (with physeal defect and transplanted MSCs) and left femur (with physeal defect without transplanted MSCs) as well as for differences in length and angular deformities before MSCs transplantation and after euthanasia. The values were statistically analyzed using the Wilcoxon matched-pairs test; STATISTICA (data analysis software system), version 7.1 (StatSoft, Inc. 2005).

Results

There were no complications during collection of bone marrow blood from iliac wings. In all ten pigs, we successfully cultured MSCs allotransplant in required amounts. In all cases, the required count of MSCs in implant was 2×10^6 cells. There were no anesthesia and post-operative complications in any of the animals. The wounds were healed *per primam intentionem*. Immunophenotyping indicated more than 95% of the cells were positive for CD29, CD44 and CD90 and they can be considered homogeneous. The expression of CD105 and CD147 was low.

Results of measurement of femur lengths show that left femurs with damaged distal growth zone and transplanted MSCs grew more in length ($0,56 \pm 0,14$ cm) compared with right femurs with physal defect without transplanted MSCs ($0,14 \text{ cm} \pm 0.3$ cm). Angular (valgus) deformity of left femurs with physal defect and transplanted MSCs was mild ($0.78^\circ \pm 0.3^\circ$). On the contrary, left femurs with physal defect without transplanted MSCs showed significant valgus deformity ($3.2^\circ \pm 2.5^\circ$). Differences in lengths and valgus deformity were statistically significant (5% level of significance, $p=0.018$).

Histological examination revealed that the defect was filled with hyaline cartilage in most cases. Immunohistochemical staining was then performed on hyaline cartilage, using type-II collagen antibodies (90% positive) and PAS staining (90% positive) and achieving similar results (Fig 1). Berlin blue staining detected the presence of rare iron granules in the cytoplasm of newly-formed chondrocytes (90%), which explains the origin of cells in the original MSC transplant (Fig. 2). The results of cell examination by immunofluorescence were also largely positive (80%), proving that the chondrocytes were differentiated from the transplanted MSCs which had been previously marked with CM-DiI. The bone bridge in iatrogenic-created distal physal defect of right femurs (control group) was every developed (Fig. 3).

Discussion

In our previous study, we reported on successful prevention of bone bridge formation and growth arrest using autologous MSC graft transplantation into iatrogenically damaged physis in rabbits (Plánka et al., 2007; Gál et al., 2007). The use of a bigger experimental animal is a logical step towards a model with both features and size similar to the human organism and it brings our experiment closer to clinical testing in human medicine. Miniature pig model is reasonable in terms of costs and easy to maintain. We obtained animals from certified

breeding at the Institute of Animal Physiology and Genetics in Liběchov, the only institution of this kind in the Czech Republic. The fact that FBS is necessary for culturing MSCs prior to the transplantation is the only impediment to clinical testing.

The collection of bone marrow and the isolation of stem cells are prerequisites for success. The obtained blood samples and the isolated cells respectively are, in addition to the previously described procedure, subjected to other typing methods. We select the best-defined cell colony based on the expression of positive CD markers and the absence of negative CD markers. The cultured cells were tested by means of the established positive markers for MSCs (CD29, CD44, CD90, CD105; CD147 has been recently added in several studies). Low expression, possibly lack of expression, in case of CD105 and CD147 might be explained by their tendency to reflect with increased sensitivity the contemporary state of the cultured cells. This is particularly the case of incipient spontaneous differentiation which can be induced by a number of external factors, such as a higher cell density and cell interaction.

To achieve results which would be valid and clear, it was essential to choose such a growth plate within the skeleton that has significant growth potential and contributes to bone growth. Taking these criteria into consideration, the selected portion of the physis, i.e. the growth zone of distal epiphysis of femur, seems to be an optimum choice as it is responsible for 70% of longitudinal growth of femur and it is the largest bone in miniature pigs. The lateral condyle is easily accessible in terms of surgery, facilitating a quick surgical procedure (reducing the time of general anesthesia) and resulting in low occurrence of postoperative complications.

Cultured MSCs were stimulated to differentiate towards chondrocytes as per the protocol (Miura, 2002) in α -MEM medium with the supplement of human recombinant TGF- β 1. The culture medium was supplemented also with 1% ITS, dexamethasone and ascorbate-2-phosphate which are commonly used ingredients of culture and differentiation medium (Li et al., 2005; Lisignoli et al., 2005; Indrawattana et al., 2004; Quintavalla et al., 2002). In some

studies, MSCs were successfully differentiated using TGF- β 3 (Lee et al., 2004; Chen et al., 2004), in some cases combined with BMP-6 or IGF-1 (Indrawattana et al., 2004). As the role of these factors and accurate timing of their application is not completely clear, we differentiated the MSCs using the TGF- β 1 alone, whose application is commonly recognized method of influencing differentiation of mesenchymal stem cells toward chondrocytes (Lisignoli et al., 2005; Li et al., 2005; Hui et al., 2005). In our study, we used TGF- β 1 (in concentration of 100 ng/ml) to affect MSCs in medium for 30 minutes (Miura et al., 2002). Today, higher concentrations of TGF- β 1 are recommended (Lisignoli et al., 2005); Hui et al. (2005) used TGF- β 1 even in concentration of 1000 ng/mL.

In our previous study, a different type of scaffold, based on tissue adhesive (0.12 ml solution of human protein for Tissucol, Tissucol®Kit Baxter), was used. This scaffold had a gel structure and following its transplantation into an iatrogenic defect in the lateral section of the distal growth zone of femur, it was necessary to close the external entry of the defect using bioceramics. It was the only way to prevent the scaffold and MSCs from travelling outside the defect. In this study, we used a solid scaffold based on collagen and chitosan which retained its shape of a compact cylinder after implanting it with MSCs and we were able to fix it in the created defect by means of the press and fit technique. In the future, the exact shaped scaffold after the MR can be created (Krupa et al., 2007).

When selecting the method to create the bone bridge iatrogenically, we built on the results of our previous study (Gál et al., 2002, Varga et al., 2007) and the experimental study of Janarv et al. (1998). We believed that this tried and tested method, causing damage to more than 9% of the growth plate area, was highly dependable. For this reason, we used a screw of 6 mm in diameter.

The proper clinical verification of the impact of preventive MSC transplantation into the defect in femoral distal physis on longitudinal bone growth and on the degree of incurred

valgus deformity was based on measuring these quantities from radiographs of femur in craniocaudal projection. The femoral length was measured as the distance between the femur head tip and the tangent of the most distant point of femoral condyle (Janarv et al., 1998), while the degree of valgus deformity of distal femur was measured as the angle between the long axis of the bone and the vertical to the tangent connecting femoral condyles. We expected that the left femur with a defect in the lateral section of the growth plate will be, due to preventive transplantation of MSCs, longer and less valgus deformed (or entirely without valgus deformity) than the right femur with the same physis defect, but without MSC transplantation (i.e. a bone bridge bridging the physis). This hypothesis was proven by measurement performed by three independent observers.

Radiological examination is readily accessible and allows determining with a relatively high accuracy both the length of the x-rayed bone and its possible angular deformity. The method of measuring the femur length was based on Janarv's original study (Janarv et al., 1998), whereas the measuring of the extent of valgus deformity had been consulted with specialists on descriptive geometry for the needs of earlier studies of the authors (Gal et al., 2007; Planka et al., 2007).

Histological examination of prepared specimens was carried out by a pathologist, using staining with haematoxylin and eosin. The tissue filling the iatrogenically-induced defect was examined under a microscope. In case of discovering cartilage, a macroscopic view was applied to determine whether it was hyaline cartilage or fibrocartilage. Potential signs of an immune reaction against the transplant observed in the body of the experimental animal (Graft versus Host Disease) were also evaluated. The site of defect filling was checked for necrosis and sequestration and lymphocyte infiltration was assessed. We can conclude that no such signs were found in any of the experimental animals. To detect hyaline cartilage properties, immunohistochemical examination for type-II collagen antibodies was chosen with regard to

available literature. All similarly-oriented studies consider this examination as a crucial indicator confirming the positive outcome of treatment. We also used PAS staining which gives a specific colour reaction with both neutral and acid mucopolysaccharides found in the intercellular substance of the cartilage. Some authors refer to alcian blue staining which, however, stains only acid mucopolysaccharides. Safranin O staining and type-X collagen antibodies were not opted for since the above-mentioned methods are sufficient to verify the results of our experiment and since the two methods are rather costly.

The results of Pearls reaction were positive, showing the detected iron particles coloured in blue (combined with Berlin blue). Considering the length of observation period (from surgery until euthanization), we expected that the originally phagocytosed grains of iron nanoparticles (Resovist) will be hardly detectable as they are subject to gradual division into daughter cells. The aggregation of the blue pigment might indicate false positivity, for example in case death of the original cells occurs and they are phagocytosed by the macrophages which are present. However, our cells with noticeable grains of iron obviously merged with viable cartilaginous tissue without signs of necrosis. To further confirm their origin, we used immunofluorescence with CM-DiI which is deposited on the membrane of MSCs during the first staining. This method of staining is also subject to quick division into daughter cells and thus the detection of the pigment using a fluorescence microscope was only localized.

The results of this experimental study in pigs confirmed our hypothesis that transplantation of autologous mesenchymal stem cells into experimental physal defect can prevent bone bridge formation and thereby bone shortening and angular (valgus) deformity. The procedure can promote healing of damaged growth plate by differentiation of transplanted MSCs into chondrocytes.

Conclusions

The MSCs which we transplanted differentiated in vivo into functional chondrocytes of the growth plate in the form of hyaline cartilage. Preventive transplantation of MSCs in our experiment prevented development of angular deformity and eliminated growth arrest of femur in physeal injuries in pigs.

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Pictures

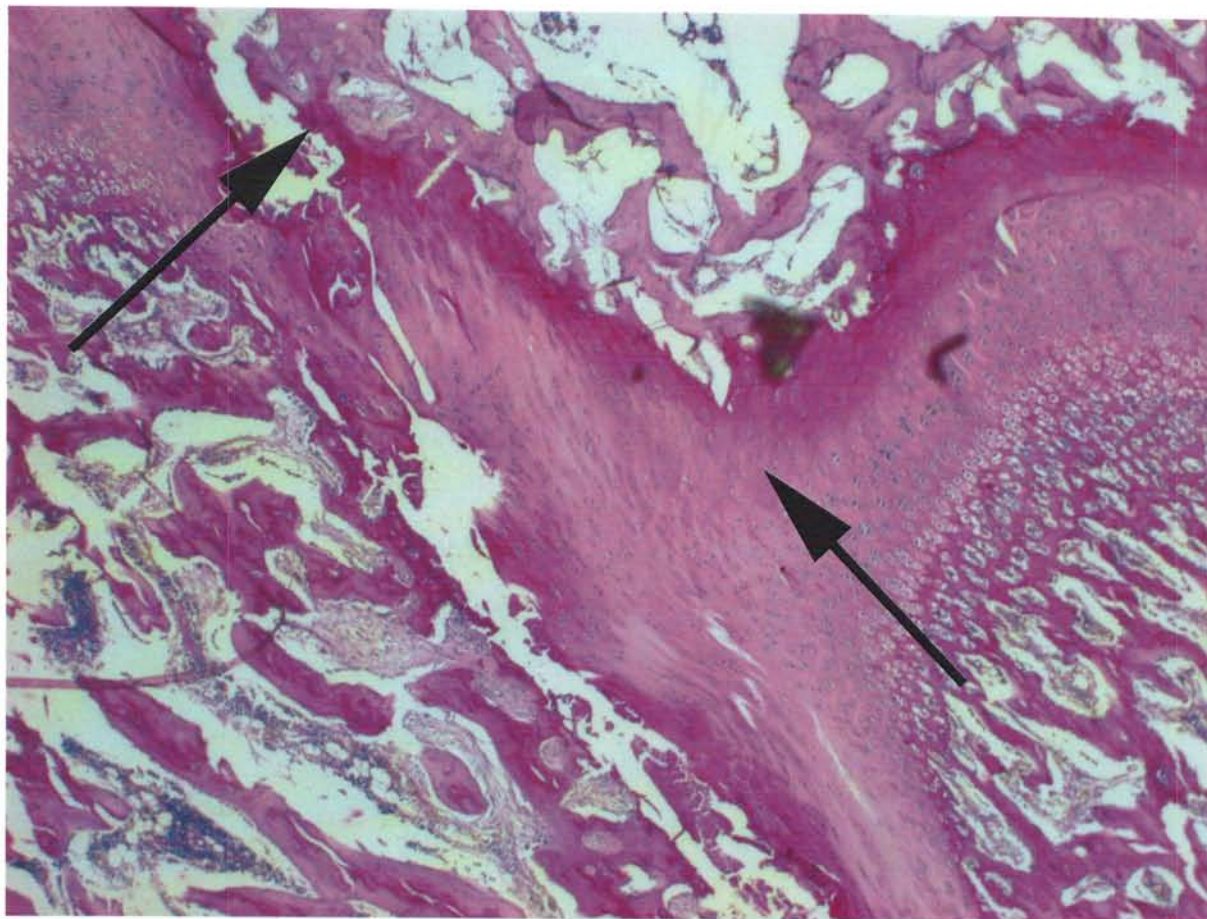


Figure 1: Histological examination of the left distal femoral physis in miniature pigs after autogenous MSCs transplantation (PAS, 40 x magnification – Pig 8) - arrows indicate the borders of iatrogenic created defect of physis, between them is the new cartilagenous tissue

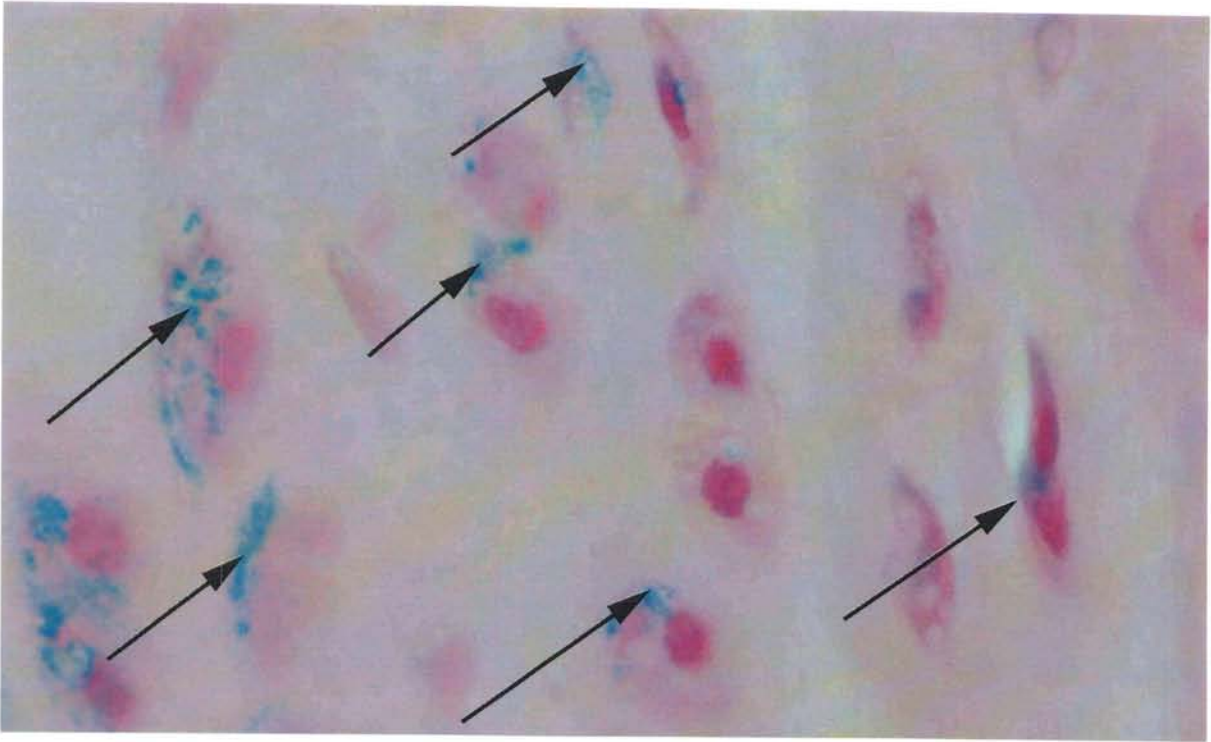


Figure 2: Resovist granule (arrows) in the chondrocytes cytoplasm (Fig 2) differentiated from MSCs – Pearls reaction (400x)

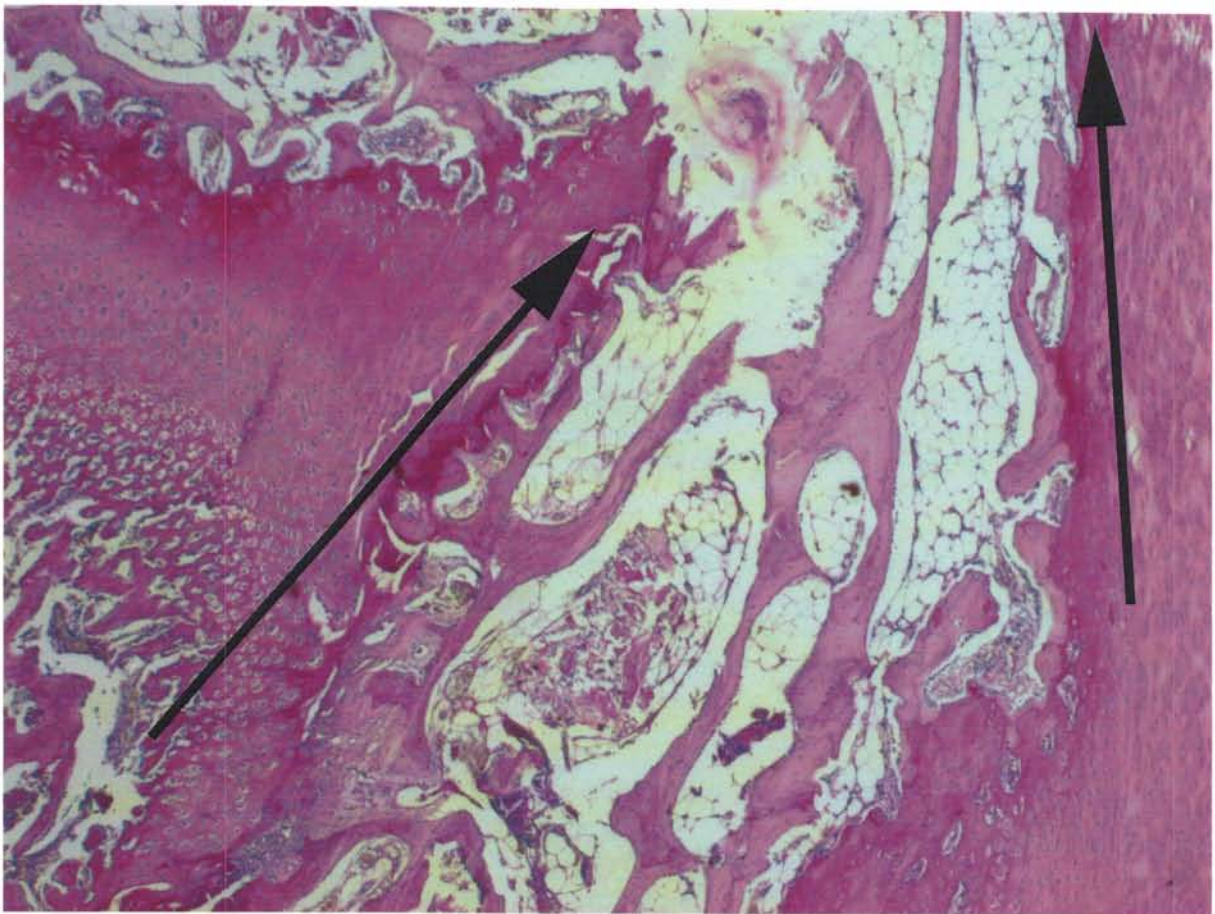


Figure 3: Histological examination of the right distal femoral physis in miniature pigs after autogenous MSCs transplantation (PAS, 40 x magnification – Fig 7) - arrows indicate the borders of iatrogenic created defect of physis, between them is the bone bridge