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A modified parapatellar approach for the creation of osteochondral defects in sheep

Maria Teresa Oliveira *, José Caeiro Potes *, José Lopes de Castro **, Alfredo Franco Pereira **, João Fragoso *, Joana da Costa Reis *

* *DMV, ECT, ICAAM Universidade de Évora, teresoliveira@uevora.pt , jacpotes@uevora.pt , jfragoso@uevora.pt, jmfcr@uevora.pt ; Pólo da Mitra, Ap. 94, 7002-594 Valverde, Évora, Portugal;*
** *DZ, ECT, ICAAM, Universidade de Évora, jcastro@uevora.pt, afpereira@uevora.pt ; Pólo da Mitra, Ap. 94, 7002-594 Valverde, Évora, Portugal;*

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Abstract

Osteoarthritis is a problem of great social and economic importance in elderly populations, mostly in developed countries. Current treatments aim to relief the clinical signs and slow the disease development, rather than cure it.

Beyond this point, cartilage regeneration has recently received much attention from bioengineering industry, mostly because it's acknowledged that early treatments of osteochondral defects (OCD) are crucial for slowing or even preventing the chronic development of OA.

The sheep is considered a promising large animal model for the testing of bone implant materials because of its potential to support preclinical translation. Several surgical techniques for the creation of the osteochondral defects have already been described. However, some use the classical medial parapatellar approach to the medial condyle of the femur, which is considered unsafe due to its high risk of posterior patellar luxation and the development of secondary osteoarthritis. This will potentially interfere with the biological and biomechanical response of the osteochondral unit to biomaterials.

The aim of this study was to develop a modified medial parapatellar approach to the creation of osteochondral defects in sheep to further test novel biomaterials and scaffolds, with the goal of favouring early weight bearing. In order to do so, all sheep underwent medial arthrotomy to access the left femoral condyle. The limb was flexed to allow access to the centre of the medial condyle and drilling of the defect without the disruption of the oblique medial vastus muscle, thus reducing postsurgical morbidities. Early loadbearing was observed in all animals and kept through the implantation period..
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Keywords: osteochondral defect; , knee; modified parapatellar approach; animal models

1 Introduction

Osteoarthritis (OA) is a problem of great social and economic importance in elderly populations, mostly in developed countries. Furthermore, OA is also the most frequent chronic musculoskeletal disorder in pets and horses, causing decreased levels of activity and life quality, and resulting in substantial financial costs **[1]**. Therefore, investing in this

[a]*****Corresponding: Maria Teresa Oliveira*
Email Address: teresoliveira@uevora.pt

area may contribute to the development of novel therapies both for humans and animals, with an important economic and social impact.

OA is a dynamic and slowly progressive condition that affects symptomatically up to 28% of the human population aged over 60 years **[2]**. Additionally, 20% of dogs over 1 year of age **[3]** , sport horses at early ages **[4]** and older horses **[5]**, among other species. Joint injuries that induce incongruity, instability, abnormal loading or malalignment may lead to OA. OA main features are the diffuse loss of articular cartilage, exposure of subchondral bone, local chronic inflammation and secondary periarticular bone proliferation. Albeit OA can affect individuals of all ages or gender, there are some known predisposing causes of joint chronic inflammation that will favour secondary OA (e.g. aging, overweight, osteochondrosis). In veterinary medicine is also recognized some breed predisposition to primary OA (e.g. Labrador Retriever) **[6]**.

Currently there's no cure for OA. Standard treatment aims at slowing its progression, providing pain relief, and improving quality of life. The multimodal managing plan includes: dietary manipulation and body weight control, physical therapy, anti-inflammatory and analgesic drugs, disease-modifying osteoarthritic drugs, nutraceuticals, and surgery **[2]**, **[7]-[9]**.

Beyond this point, cartilage regeneration (cell-based therapies and scaffold-based cell delivery) has recently received much attention from bioengineering industry, mostly because it's now acknowledged that early treatments of osteochondral defects (OCD) are crucial for slowing or even preventing the chronic development of OA. Nevertheless, several authors pointed out that tissue engineering treatments are far from ideal, achieving varied levels of success, not always ensuing tissue regeneration **[10]**, **[11]**.

Cell-based therapies are mainly used in human medicine and equine clinics **[7]**, **[8]**, **[12]**, **[13]**; however, they aren't very popular among general practices due to its costs, unfeasibility, efficacy, and safety issues. The primary cell sources include embryonic stem cells and mesenchymal stem cells (MSCs). Major disadvantages comprise difficulty to treat large lesions, donor site morbidity, complex surgical techniques (e.g. subchondral bone microfracture) **[7]** and MSCs dedifferentiation into fibrocartilage.

On the other hand, with the development of biomaterials and scaffolds (that serve as a frame for the chondrogenic and osteogenic differentiation of MSCs) the use of cell-based therapies in OCD could become unnecessary **[14]**. The key properties for their success are high porosity, biocompatibility, and certain mechanical properties (e.g. permeability, adhesiveness and bioactivity). Finally, they should be injectable, to enable minimally invasive surgery. There are natural and synthetic biomaterials that can be used alone or combined. The main advantage of natural biomaterials (e.g. collagen, fibrin, hyaluronan or chondroitin sulphate, chitosan and alginate) is their ability to mimic extracellular matrix thus facilitating cell adherence and differentiation, while exhibiting optimal biocompatibility and biodegradability; limitations include the requirement of purification protocols, less mechanical strength and difficult manipulation **[13]**. Synthetic biomaterials [such as poly (α-hydroxy esters) and bioceramics] offer high primary stability and are easier to handle, being also effectively integrated within the host tissues **[13]**.

Regarding the different models available, Orth and Madry **[10]** summarized 31 translational investigations, comparing between different species (small and large animals) and between TE techniques and defect sites. Moreover, the impact of some factors over the ability of the subchondral bone plate to advance towards the joint line was acknowledged as of increasing relevance for translational models of osteochondral repair in TE. These factors include, for example, the altered subchondral bone/articular cartilage crosstalk, neo-vascularization, and altered biomechanical forces at the defect site **[15]-[17]**. Finally, several authors refer the sheep as a promising large animal model for the testing of bone implant materials because of its potential to support preclinical translation both by offering similarities in the repair capacity of articular cartilage defects and by offering similar biomechanical properties including long bone dimensions and body weight to humans **[15]**, **[17]-[19]**. Several surgical techniques to the creation of the osteochondral defects have been described in large animal models **[15]-[17]**. The classical medial parapatellar approach to the medial condyle of the femur is considered by some authors unsafe due to its high risk of posterior patellar luxation and the development of secondary osteoarthritis [1], [20].

The aim of this project was to develop a modified parapatellar approach for the creation of load-bearing osteochondral defects in the sheep's medial femoral condyle that would allow the study of the biological and biomechanical response of the osteochondral unit to biomaterials.

2 Experimentation

All animal handling and surgical procedures were conducted according to European Community guidelines for the care and use of laboratory animals (Directive 2010/63/UE) and after obtaining approval from the national competent authorities. Twenty-four skeletally mature female Merino sheep with an average body weight of 51.0±6.4 kg and an average age of 6.4±1.2 years, were divided into three groups: group A (n=8), control group, where the osteochondral defect was left empty; group B (n=8) and group C (n=8), experimental groups where a ceramic and a polymeric scaffold were inserted, respectively. One defect per animal was performed in the medial condyle of the left femur.

Premedication was with subcutaneous atropine 0.7 mg/kg, intramuscular xilazine 0.05-0.1 mg/kg, intravenous butorphanol 0.01 mg/kg and subcutaneous carprofen 2 mg/kg; induction was achieved with intravenous thiopental sodium 5% 5-10 mg/kg and maintenance with isoflurane 1%–2% under spontaneous ventilation. After induction the sheep were positioned in right lateral recumbence with the left hind limb in physiologic extension fixed to the surgical table. The surgical field was prepared with povidone-iodine solution and alcohol at 70°, and the anaesthetic monitoring equipment connected. Orogastric intubation was performed.

All sheep underwent medial arthrotomy to access the left femoral condyle. An innovate parapatellar technique avoiding the lateral luxation of the patella, previously developed in an *ex vivo* model, was the chosen approach to create a loadbearing osteochondral defect in the medial femoral condyle. A skin incision was performed extending from the medial side of the tibial tuberosity to the immediate proximal side of the patella. At this point, the limb was temporarily flexed. Subcutaneous tissue was debrided, and the medial patellar retinaculum incised to expose the joint capsule (*Fig. 1a*). An incision was made over the medial side of the joint capsule to accede to the medial condyle. The incision of the oblique medial vastus muscle was prevented. With the limb in flexion, an osteochondral defect with 7 mm of depth at the periphery and 9 mm of depth at the centre was manually drilled in the centre of the medial condyle, approximately 1.5 cm apart from the femoral trochlea. This last procedure was performed under the guidance of a drill depth gauge and a drill stop to standardize the defect size (*Fig. 1b*). The defect was then rinsed with physiologic saline and, when required, the scaffold inserted (*Fig. 1c*). Limb extension was restored, and the joint capsule, retinaculum, subcutaneous tissues and skin were sutured, following this order.

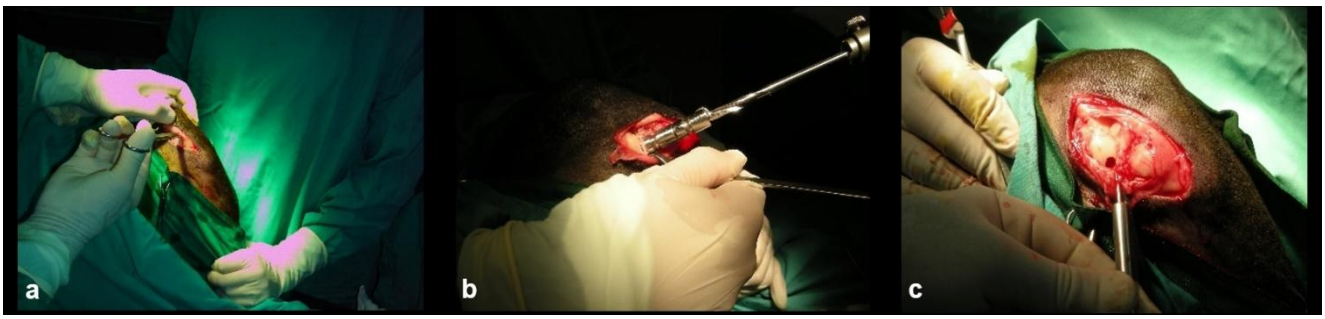

Fig. 1 Some surgical steps: a) incision of the retinaculum with the limb flexed; b) manual drill with drill stop key; c) defect in the medial condyle.

Upon recovery from anesthesia, the sheep were moved into a pen, inside the Veterinary Hospital of the University of Évora, and treated with amoxicillin and clavulanate acid, carprofen, and butorphanol, for 7 days. Fifteen days postsurgery, a fluorochrome (calcein green) was subcutaneously injected, and sheep were released into the pasture. Another fluorochrome (alizarin complexone) was subcutaneously injected 2 weeks before sacrifice. After 6 months of implantation time, the animals were sacrificed by pentobarbiturate intravenous injection. After sacrifice, soft tissue was extracted from the knee and the samples were cut with the help of a bone saw, preserving the implant and the surrounding

areas, to fit the micro-CT chamber. The samples were collected and stored immersed in 4.0% formaldehyde in phosphate buffered saline for two weeks, for fixation.

The biological response and material integration were assessed by conventional radiography, micro-computerized tomography (micro-CT), and histological and immunohistochemistry studies. After macroscopic inspection, all the samples underwent micro-CT scanning (Skyscan 1174, Kontich, Belgium). The samples were removed from 4% formaldehyde, rinsed with distilled water and coated with Parafilm M® (Sigma Aldrich, Missouri, USA), to avoid sample dehydration. Subsequently, the condyles were posed in a rotation stage fixed by commercial play-dough. Scans were performed with 50-kVp, 800-µA, and a 1-mm aluminum filter. The pixel size was 62.08, exposure time 2,200 ms, rotation step 0.8°, full rotation over 360°, with 2 average frames per image. Each condyle went through one scan, over approximately 55 minutes, assuring the imaging of the condyles containing the osteochondral defects, implanted or not, comprising 400 cross-sections. The cross-section images were reconstructed using N-Recon software (Skyscan, Kontich, Belgium). In the analysing software (CTAn, Skyscan, Kontich, Belgium) one volume of interest (VOI) was created – VOI_defect, which consisted in a circular VOI with approximately 9 mm of diameter, centred in the bone defect; its first cross-section was determined to be the one where the defect's entry point was completely surrounded by trabecular bone, then it was extended for 150 identical cross-sections, thereby creating a VOI that contained the bone defect/ plug and surrounding trabecular bone. The following parameters were evaluated: trabecular bone mineral density (BMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), trabecular pattern factor (TbPf). Uniform threshold method was applied. For histomorphometry the femoral sections were fixed in 4% paraformaldehyde and embedded in methylmethacrylate resin. Sections were obtained on a diamond saw microtome with an average width of 70 µm and stained with Giemsa Eosin. The bone-implant interface was assessed following the guidelines approved by the American Society for Bone and Mineral Research **[21]**. For immunohistochemistry (collagen I and II) and histochemistry (Masson and Mallory trichromes), bone sections were decalcified in 5% formic acid solution, embedded in paraffin and cut in 3 µm sections. Markers of osteogenic differentiation (osteopontin, osteocalcin and collagen type I) and osteoclasts marker (TRAP) were studied in the subchondral bone region.

3. Results and discussions

The results here presented comprise surgical and post-surgical in vivo results of the applied surgical model. For confidentiality reasons related to the materials, contractually bound, the post-mortem results displayed are only from the control group, although the same procedures were performed in all groups.

A range of large animal models have been investigated for the assessment of cartilage repair, including dogs **[22]-[24]**, pigs **[25]-[29]**, sheep **[14]**, **[30]**, **[31]**, goats **[32]-[35]** and horses **[12]**, **[36]**,**[37]**.

The physiology of articular cartilage, both in health and damage, strongly depends on the biomechanical environment. Chondrocytes recognize physical signals from their environment through a variety of mechanisms, including ion channels and integrin-mediated connections to the extracellular matrix that involve membrane, cytoskeletal and intracellular deformation **105[38]**. The restoration of biomechanical and biotribological functions, setting the correct stress-strain distribution and environment for tissue repair, is critical **[39]**.

In sheep, the average peak axial tibio-femoral contact forces are estimated as being of 2.1 times the body weight (BW), with only small medio–lateral and antero–posterior shear forces, averaging 0.7 BW. Average knee flexion angles ranging from 49º to 70º were observed in a previous study **[40]** and individual and breed-related variation are expected. Peak tibio-femoral contact forces in humans are higher, ranging from 2.8 to 3.8 times BW during walking and up to 6.2 BW during stair climbing **[41]**, but although there are differences between both species, forces are comparable, and the joint anatomy is close **[42]**. Additionally, the ovine stifle joint presents cruciate ligaments very similar to humans' and large menisci, along with a similar lateral collateral ligament (LCL) complex, amongst other structures. This allows surgical training and the use and development of surgical prosthetics and devices **[43]**, **[44]**.

It is therefore important to consider load transfer when designing surgical pre-clinical animal models that address cartilage and osteochondral repair. A choice was made to create a defect in the medial condyle in alternative to the

trochlea, since clinically most defects occur on the weight-bearing medial condyle of the femur, and the trochlea is only partially loaded. A unilateral model without postsurgical joint immobilization was chosen due to welfare issues.

The *in vivo* surgical procedure was performed based on literature review and the surgeon's own experience **[1]**, **[14]**, **[44]**. The disruption of the oblique medial vastus muscle, as preconized in the classical medial parapatellar approach **[44]**, was avoided, reducing the postsurgical morbidity and the possibility of complications like the luxation of the patella and osteoarthritis **[1]**, **[20]**.

All sheep recovered well and rapidly stood up after surgery, immediately supporting weight in the intervened limb. Yet, in the immediate postsurgical period a lameness of grade III/IV (out of V) was patent. After the postsurgical period all animals were released to pasture with no evident signs of lameness (grade I-II). The *in vivo* procedures were successful with all animals completing the 6-month implantation period with obvious signs of welfare, such as an average weight increment of 6.37±4.13 kg (Table 1), confirming consistent feeding and foraging behaviours. A long implantation study as the one chosen is necessary to gain confidence in the extent of success in the repair and regeneration of articular cartilage, including interface with adjacent cartilage and subchondral bone, as well as the opposing articular surface.

Table 1. Characterization of the sheep

Group	Age (years)	$Weight_{t0}$ (kg)	$Weight_{t1}$ (kg)
A	6,4±1,2	50,3±2,4	53,0±4.3
B	6,6±1,2	51,9±5,3	60.5±5.8
C	6,3±1,4	50,9±7,6	58.6±7.3

$Weight_{t0}$: presurgery weight; $Weight_{t1}$: weight at sacrifice

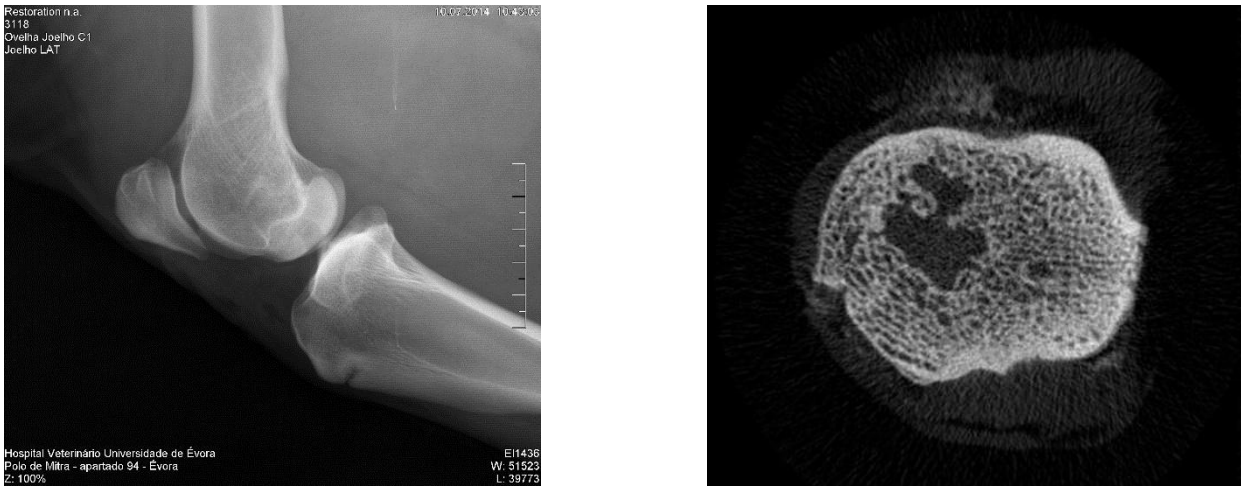

Fig. 2 a) immediate postsurgical plain x-ray showing the load-bearing position of the OCD (sample from the control group); b) postmortem micro-CT cross-section image of the OCD, showing the defect with scarce newly formed trabecular bone

Postsurgical patellar luxation was not observed in any animal. It is also important to emphasize that constant anesthetic monitoring by a qualified veterinary enabled prompt intervention when necessary.

Ancillary imaging, like x-ray and micro-CT, were crucial in offering visualization of the osteochondral defects and the biomaterial integration at the time of the surgery and after the sacrifice (Fig. 2).

At the end of the *in vivo* study, micro-CT scanning was performed.

The control group samples showed areas of defect yet to be filled in by trabecular bone. These observations are illustrated by *Fig. 2b) and 3b).*

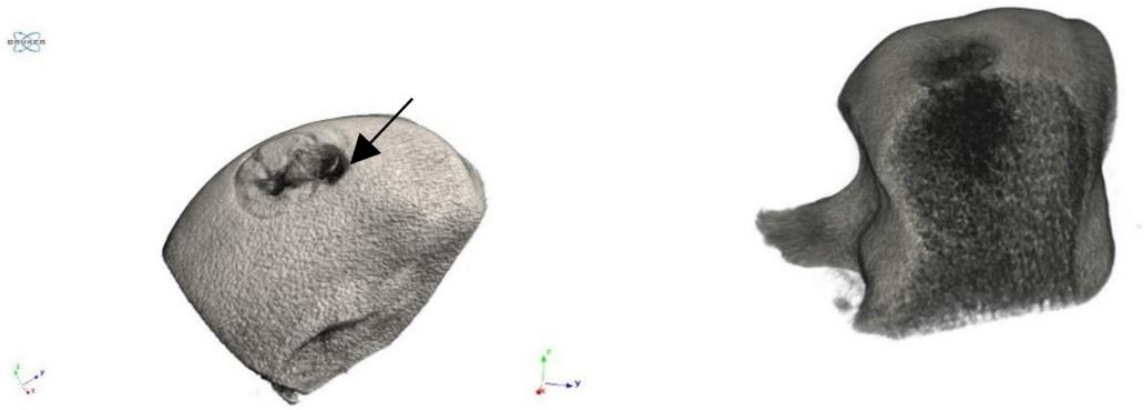

Fig. 3 Micro-CT 3D reconstruction images of the condyle show a) a depression where the OCD was created 6 months early and the site of post-mortem RT-PCR's sample collection (arrow), and b) the disruption of the trabecular bone structure in a part of the original defect area

Results of 3D histomorphometric analysis are summarized in Table 2. Histomorphometric analysis allowed the quantitative comparison between the control and the experimental groups.

Table 2. Histomorphometric results from control group

Group	BMD	BV/TV	Tb.Th	Tb.Sp	Tb.N	TbPf
	g/cm^3	mm^2/mm^3	mm	mm	1/mm	1/mm
A	0.53±0.08	69.28±13.81	0.80±0.34	0.68±0.69	0.95±0.25	5.33±5.01

Trabecular bone mineral density (BMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), trabecular pattern factor (Tb.Pf)

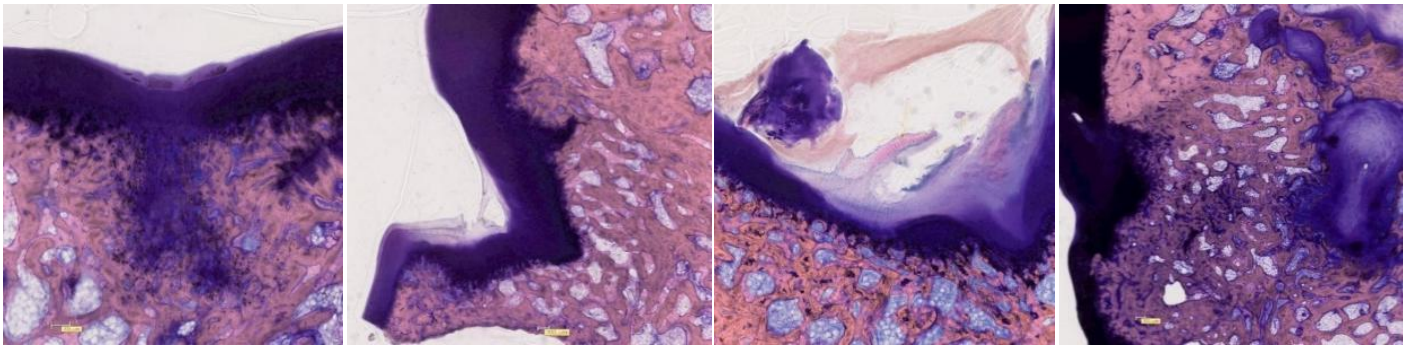

Fig. 4 Control samples' sections. a) clear disruption sites of the cartilage and the subchondral bone (2.5 X magnification); b) newly formed cartilage after a 6-month implantation time (1.25X magnification); c) predominant scar fibrous tissue during the healing process (2.5X magnification); d) defects in the bone structure filled by conjunctive fibrous tissue (1.25X magnification).

For histology and immunohistochemistry, samples were processed, and sections prepared and recorded as described in the experimental section. The defect areas were visible. On the majority (six out of eight) of the sections of the control group, a depression on the articular cartilage surface was evident where the defect had been. However, in all the sections there was continuity of the articular cartilage, even if there was also cicatricial fibrous tissue on the top (*Fig. 4a-c*). There were evidences of changes in the subchondral bone trabecular structure in all samples and in four of them considerable gaps were left in bone (*Fig. 4d*).

4. Conclusions

A new ovine model for parapatellar approach has been developed. The surgical technique described, first developed *ex vivo*, is reproducible and safe under physiological loads.

The model is innovative in the approach, wherein the intra-operative flexion of the limb allows to create the defect avoiding the disruption of the oblique medial vastus muscle, thereby reducing postsurgical complications such as recurrent patellar luxations and osteoarthritis and allowing early limb loading.

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