HISTOLOGICAL SCORING SYSTEMS IN THE CARTILAGE REPAIR OF SHEEP

Gullberg, S.¹, Simaiová, V.², Holovská, K.³, Luptaková, L.⁴
Kolčvek, F.⁵, Varga, M.², Petrovová, E.²

¹Skövde Djurklinik AB, Bäckaskogsvägen 16, 541 34 Skövde
Sweden

²Institute of Anatomy

³Institute of Histology and Embryology

⁴Institute of Biology, Zoology and Radiobiology

⁵Equine Clinic, University of Veterinary Medicine and Pharmacy in Kosice
Komenského 73, 04181 Kosice
Slovakia

eva.petrovova@uvlf.sk

ABSTRACT

Researchers around the world use histological analysis to provide the most detailed morphological information of articular cartilage repair and it predominantly relies on the use of histological scoring systems which are important tools for valid evaluations. Due to hyaline cartilage complex structure and avascular nature, damaged cartilage does not heal spontaneously and it is still a challenge to regenerate and restore its tissue function. The aim of this study was to investigate the quality of regenerated cartilage by using three different histological scoring systems; O'Driscoll, Pineda and Wakitani which are all classic scores described for such animal studies. We used an *in vivo* ovine model in which a full thickness chondral defect was created and then implanted with

the biomaterial (polyhydroxybutyrate/chitosan; PHB/CHIT). The results of this histological analysis demonstrated that the cartilage repaired tissues received scores indicating that the majority of the regenerated tissue resembled hyaline-like cartilage. After six months of repair the regenerated cartilage showed characteristics like good surface continuity, uniformed stained extracellular matrix, clearly visible zones and cellular proliferation. In conclusion, this study may be used to investigate and improve the regenerative capacity of hyaline cartilage in preclinical models and it also sheds further light on both the evaluation and methods used for the regeneration of damaged cartilage.

Key words: animal model; articular cartilage; cartilage repair; histology; regeneration; scoring system

INTRODUCTION

Cartilage is a flexible, sustainable form of tissue composed of cells called chondrocytes and a highly specialized extracellular matrix. The extracellular matrix has unique biomechanical properties and is composed of ground substance and fibers. The matrix contains high concentrations of glycosaminoglycans (GAG) and proteoglycans (PG) and these can interact with elastic and collagen fibres. The composition of the matrix components and cells can create variations and produce three different kinds of cartilage adapted to local biochemical needs [10]. Hyaline cartilage can be commonly found in the ribs, nose, larynx, and trachea. Hyaline cartilage on the articular surfaces of bones (the surfaces in joints) is called articular cartilage (AC). Healthy AC provides a smooth, cushioning and low friction surface for joints [20], and is composed of four layers based on the structure of the extracellular matrix and cell morphology: a) superficial zone, b) intermediate zone, c) radial zone, and d) calcified cartilage zone [14]. It is known that AC can tolerate considerable intense and repetitive stress but it has a limited ability of repair. This inability to heal is attributable to a few factors, such as the chondrocytes immobility, limited ability of mature chondrocytes to proliferate and the avascularity of cartilage. Degradation of AC result in osteoarthritis, even minor injuries of AC might lead to progressive damage and osteoarthritic joint degeneration. It is one of the global leading causes of significant pain, disability and immobility [12, 13]. Globally, osteoarthritis affects around 3.3 % to 3.6 % of the world population. It is the 11th most debilitating disease around the world since it causes moderate to severe disability in 43 million people [3, 4]. In attempts to regenerate or repair AC many methods has been developed by researchers and surgeons whose goal is to create and restore the function of hyaline cartilage.

Nowadays tissue engineering is able to create live tissue and is used to replace or improve biological function; it can be done by the usage of combinations of cells, biomaterials, biochemical and physio-chemical factors. The methods used for cartilage repair and regeneration do not perfectly restore cartilage but there are very promising results seen in repairing cartilage from traumatic injuries or cartilaginous diseases.

At this time, there are several different clinical methods available for cartilage repair which includes: autologous

chondrocyte implantation (ACI), bone marrow stimulation, osteoarticular transfer system or mosaicplasty, osteochondral plug transplantation, penetration of the subchondral bone, matrix-induced autologous chondrocyte implantation, microfracture and most recently the next generation of ACI which involve scaffolds or cell-seeded scaffolds. There has recently been a significant expansion in biomaterial technologies, cell sources, scaffolds, molecular and genetic manipulations for the creation of functional tissue replacements to treat damaged cartilage [13]. It has been shown that implantation of acellular biomaterials significantly enhances the regeneration of cartilage compared to natural healing. Some advantages that acellular biomaterials offer includes: no costs for cell cultures, lack of donor-site morbidity, not as many regulatory issues, and one-stage surgical procedures.

Natural biomaterials used *in vivo* includes chitosan, collagen, hyaluronic acid and alginate. Also, synthetic biomaterials exists such as polyvinyl alcohol, poly(lactico-glycolic acid) and polycaprolactone. Multi-layered biomaterials are made to combine the beneficial properties of these materials, for example β -tricalcium phosphate-hydroxyapatite/hyaluronateelocollagen and type 1 collagen-hyaluronic acid-fibrinogen hydrogel. These biologics are natural factors which induce proliferation and differentiation of cells [19]. The goal of cartilage repair techniques is to restore the function to the injured tissue as well as reduce pain.

To evaluate the success and outcome of cartilage regeneration one of the most important measures is the histological quality of the cartilaginous tissue. Nowadays a variety of histological scoring systems are used to evaluate and describe the quality of *in vivo* repaired tissues. Histological analysis is an excellent tool for a valid and objective evaluation of repair tissue dependent on the use of established scoring systems such as: O'Driscoll, Pineda and Wakitani. These systems are among the most commonly used for experimental cartilage repair and histological analysis is considered the gold standard method for evaluation of cartilaginous repair. The O'Driscoll score was the first histological grading system for articular cartilage defects and it was used to assess the effects of periosteal grafts in rabbits in the treatment of full thickness chondral defects [16]. This scoring system includes parameters such as: cellular morphology, safranin-O staining of the matrix, surface regularity, structural integrity, thickness, bonding to the

adjacent cartilage, freedom from cellular changes of degeneration (hypocellularity), chondrocyte clustering, and freedom from degenerative changes in adjacent cartilage [17]. When using the O'Driscoll score it is one of a few systems that allows for the assessment of the integration of the repaired tissue with its surroundings. Nevertheless, these extra added points do not significantly affect the final score and the scoring system is often used in modified versions [21]. The score value in this complex system ranges from 0-24 points where 0 is no signs of cartilage repair and 24 is complete regeneration, the maximum points value for one parameter range between 2—4 [17].

Stephen Pineda and co-workers introduced a semi-quantitative scale for histologic grading, composed of four parameters in order to evaluate the natural healing process of articular cartilage repair. A rabbit model was used by drilling defects into the articular cartilage of the knee and the samples stained with Safranin-O staining [18]. The simple Pineda scoring system scale contains four parameters; filling of the defect in percentage, reconstitution of the osteochondral junction, staining of the matrix, and cell morphology. The score value in the Pineda system ranges from 0—14 points, where 0 is complete regeneration and 14 is no repair, the maximum point values for one parameter range between 2—4. It means that the system is the inverse in which a low score value represents a good repair response [17].

The Wakitani system is an elementary scoring system developed by Shigeyuki Wakitani and co-workers in 1994. The system was used for histological evaluation of toluidine stained samples from a study where the authors investigated the effect of periosteum or bone marrow derived mesenchymal cells for the repair of large, full-thickness defects of articular cartilage. Rabbits were used as the animal models in which a chondral defect was created in the medial femoral condyle [24]. The Wakitani scoring system scale contains five parameters: cell morphology, matrix staining, surface regularity, thickness of cartilage, and integration of donor with host cartilage. The score values range from 0—14 points where 0 is complete regeneration and 14 is no repair, the maximum point values for one parameter range between 2—4, similar to the Pineda score [17].

The aim of this study was to evaluate cartilage repair after the creation of a full thickness defect and implantation of biomaterial in the sheep stifle joint by using different established histological scoring systems (O'Driscoll,

Pineda, Wakitani). These scoring systems histologically investigate the regenerated articular cartilage after implantation with a polyhydroxybutyrate/chitosan (PHB/CHIT) scaffold.

MATERIALS AND METHODS

Preparation of polyhydroxybutyrate/chitosan implants

The scaffold polyhydroxybutyrate/chitosan (PHB/CHIT) composite was prepared according to [9]. PHB (GoodFellow), dissolved in propylene carbonate were mutually mixed with chitosan (Sigma Aldrich) dissolved in 1 % acetic acid at a ratio equal to 1 : 1. For precipitation the same volumes of differently concentrated biopolymer solutions were used. The mixing was accomplished by using a magnetic stirrer at 400 rpm for 10 minutes, thereafter acetone was added to the suspension for the complete precipitation of the biopolymers. The final product was filtered and washed with distilled water. Subsequently it was moulded into cylindrical forms that were 10 mm in diameter and 10 mm in height and lyophilized (Ilshin) for 6 hours. Sterilization of the implants were done at 121 °C in an autoclave. Observation of the polyhydroxybutyrate/chitosan mixture appearance demonstrated a macroporous microstructure of the spongy-like biopolymer composite implanted with high fractions of irregularly shaped macropores with sizes up to 100 µm and micropores < 20 µm (Fig. 1) [23].

Fig. 1. Polyhydroxybutyrate/chitosan biomaterial

Sheep cartilage sample collection

Animals

Five clinically healthy ewes of the crossbreed Merino and Valachian were used. The age of the sheep ranged from 1.5–2 years with an average body weight of $50.7 \text{ kg} \pm 1.9 \text{ kg}$. They were housed at the Clinic for Ruminants at the University of Veterinary Medicine and Pharmacy in Kosice where they were kept in free stalls. Water was freely available and they were fed hay and concentrates. The animals got 14 days to acclimatize to their new environment before the scheduled surgery. They also underwent a standard preoperative clinical examination before being included in the study. The health status of the sheep were evaluated daily after the surgical intervention and the animals were sacrificed six months after the surgical procedure. The experimental animals from the farm PD Agro Michalovce (Slovak Republic) and the experimental protocol were approved and obtained from the State Veterinary and Food Administration of the Slovak Republic No. 3508/17-221.

Anaesthesia and postoperative care

Prior to the surgery, the sheep had been fasted, e.g. food and water were withheld for 12 hours. The sheep were premedicated with butorphanol (0.1 mg.kg^{-1} IM, Butomidor, Richter Pharma, Wels, Austria) and medetomidine 0.02 mg.kg^{-1} IM (Cepetor, CP-Pharma Handelsgesellschaft, GmbH, Burgdorf, Germany) for induction and for maintenance of anaesthesia ketamine 8 mg.kg^{-1} IV (Ketamidor,

Richter Pharma, Wels, Austria) was used. Post surgically all sheep were given NSAID flunixin meglumine (2.2 mg.kg^{-1} IM, Flunixin a.u.v., Norbrook, Newry, UK) $1\times$ daily for 7 days and broad spectrum antibiotic oxytetracycline dihydricum (20 mg.kg^{-1} IM, Alamycin LA a.u.v., Norbrook, Newry, UK) $1\times$ every second day.

Surgical process

To induce the traumatic defect in the articular cartilage of the left stifle joint, a skin incision was placed over the left lateral side from the medial patellar ligament distal to the tibial tuberosity. After incising subcutaneous tissue and the superficial fascia, the medial femoral condyle was exposed. The joint was flexed and partially luxated to allow for the creation of a full thickness defect in the articular cartilage by using a drill machine. The full thickness defect was created at an exact location in the distal epiphysis of the femur (trochlea femoris sinister) with a diameter of 6 mm and a depth of 2 mm. At the site of the defect, drilling Kirschner wires were used to reach the space of the bone marrow area and after that, the defect was filled with a biopolymer implant (Fig. 2). The sheep were sacrificed 6 months after implantation and the cartilage samples were obtained from biopsies (diameter 8 mm and a depth of 10 mm) harvested from the healing defect by using an Osteochondral Auto-graft Transfer System (OATS, Arthrex, USA). Also, biopsy removal was performed in the same way to get control samples from the original healthy cartilage but taken from the opposite side of the trochlea.

Fig. 2. Surgical procedure

- a) Inducing of articular full thickness cartilage defect; (b) Inserting of scaffold into prepared articular defect;
(c) Articular defect with implanted scaffold

Histological preparation

Hematoxylin-Eosin staining

Immediately after the animals were sacrificed the cartilage samples were dissected. Straightaway after harvesting the samples they were fixed in 10 % neutral formalin and decalcified with chelaton; after that dehydration was performed in a 70—100 % ethanol series and the next step was paraffin embedding. When embedded in paraffin, the cartilage samples were cut into 7 µm thick pieces. These sections were stained for routine histological study with haematoxylin and eosin (H&E) for the histological evaluation under the light microscope (Olympus CX23). By using this staining technique, it is possible to evaluate the overall structure including the tissue repair, cell morphology, bone cartilage interference, abnormal calcification and tidemark [5].

Alcian blue staining

The deparaffinized and hydrated sections were submerged in 3 % acetic acid solution for 3 minutes and then washed in tap water. Sections were stained with Alcian blue solution (Sigma Aldrich, USA, 1 % solution in 3 % of acetic acid, pH 2.5) and counterstained with Nuclear Fast Red solution (Merck Millipore, USA) for staining of the chondrocyte's nucleus. Afterwards the sections were washed in tap water, dehydrated, cleared and mounted in medium (Entellan, Merck Millipore, USA). This staining was used for the evaluation of the glycosaminoglycans in the matrix of the cartilage, it also displayed the cellular morphology and collagen deposition.

Evaluation of cartilage samples

The histological grading and observations were carried out on 50 articular cartilage samples, five sheep from which five slides with two sections on each slide were available for the study, meaning that 10 samples from each animal were used. These samples were acquired from a cartilage repair investigation and taken from regions across the trochlea in which full thickness chondral defects were created using an *in vivo* ovine model. These were then given scores, e.g. graded with the Pineda, O'Driscoll and Wakitani systems [16, 18, 24]. The Pineda system on a scale from 0 (complete regeneration) to 14 (no repair), the O'Driscoll system on a scale from 0 (no signs of cartilage repair) to 24 (complete regeneration), and the Wakitani system on a scale from 0 (complete regeneration) to 14 (no repair). These evaluations were done on all cartilage samples from the trochlea

under light-microscopy and the data were obtained, the samples were scored with individual scores from each system according the three different scoring systems. Before starting with the evaluation of the experimental cartilage samples it was important to become familiar by means of self-education by studying the appearance of physiological articular cartilage, fibrocartilage and degenerated cartilage. The samples were not presented to the observer in a blind order.

Statistical analysis of the data were performed using the programme GraphPad Prism 6.0. One-way and two-way ANOVA tests were used for analysis. Values of $P < 0.05$ were deemed statistically significant.

RESULTS

Histological scoring of cartilage repair

The examination of the histological sections from the trochlea with its full thickness chondral defects showed that articular cartilage regeneration was observed in some animals. The experimental group displayed evidence of newly formed repaired tissue with clearly visible zones in the defect site and hyaline-like cartilage formation demonstrated by Alcian blue and H&E staining. These histological observations of the repaired tissues were also reflected

Table 1. Results of the histologic evaluation of cartilage repaired tissue for the complex O'Driscoll together with simple Pineda and Wakitani semi-quantitative scoring systems

Score	Sheep	Mean	Range	Median total score
Pineda	1	2.2	2—3	3.06
	2	3.8	2—4	
	3	5.8	5—7	
	4	2.3	1—3	
	5	1.2	1—2	
Wakitani	1	3.4	2—4	3.36
	2	2.8	2—4	
	3	5.9	5—7	
	4	2.5	1—3	
	5	2.2	2—3	
O'Driscoll	1	14.3	14—16	14.28
	2	15.0	15	
	3	9.3	7—11	
	4	14.8	14—16	
	5	18.0	18	

Results are expressed as the mean value for each sheep

Graph 1. The mean score for each evaluated parameter scored with O'Driscoll

Graph 2. The mean score for each evaluated parameter scored with Pineda

Graph 3. The mean score for each evaluated parameter scored with

Graph 4. Results of the evaluation according the different histological scoring systems

in terms of the histological scoring. The trochlear sections that were graded with the Pineda and Wakitani scoring systems showed low scores, however the result from the O'Driscoll system revealed a higher score (Tab. 1; Graph 4). Since the samples only consisted of the defect and not adjacent cartilage, a modified O'Driscoll score was used and the parameters, bonding to the adjacent cartilage and freedom from degenerative changes in adjacent cartilage was not used in this evaluation. The trochlear defect graded with a modified O'Driscoll system on a scale from 0 (no signs of cartilage repair) to 19 points (complete regeneration) got a total mean score of 14.28 points and suggested a satisfactory result with hyaline-like tissue (Graph 1).

When the trochlear defect was graded with the Pineda system on a scale from 0 (complete regeneration) to 14 points (no repair) got a total mean score of 3.06 points, this low value represented a good repaired response. When scoring with the Wakitani system the parameter, integration of donor with host adjacent cartilage was evaluated on macroslices. Grading with the Wakitani system showed a total mean score of 3.36 points; the system was similar to the Pineda and graded on a scale from 0 (complete regeneration) to 14 points (no repair). The result of 3.36 points correlated with the Pineda score results and reflected a good repair (Graphs 2 and 3).

Assessing the data obtained by scoring with the different systems, the individual score per sheep varies when using the same system. Once the five sheep were scored with the Pineda system, the individual scores for the sheep ranged between 1.2–5.8 points. The scores for the sheep when graded with Wakitani system ranged between 2.2–5.9 points and for the O'Driscoll system, the scores ranged between 9.3–18.0 points (Tab. 1).

In the control samples harvested from healthy articular cartilage all zones were clearly visible. The superficial or tangential zone had the lowest content of PGs, since the chondrocytes synthesize high concentrations of collagen and only low concentrations of proteoglycan, thus making it the zone with the highest water content and the Alcian blue was less intense in these area. The superficial zone of the trochlea showed chondrocytes lying parallel to the joint surface with an oval shape instead of the flat elongated appearance that were usually seen. In the transitional, intermediate or middle layers round and slightly larger chondrocytes surrounded by extracellular matrix were seen. The collagen fibres were thick and arranged randomly,

the layers were also more intensively stained with Alcian blue which indicated a higher proteoglycan content. It is viewable that the cell density in this zone was lower and they were embedded in an abundant extracellular matrix. The cell density in the transitional zone was lower which indicated mature cartilage with predominantly spheroid-shaped cells embedded in an abundant extracellular matrix. The deep or radial zone consisted of large chondrocytes most of them were grouped in radial columns and the arrangement was perpendicular to the surface. Noticeable was that this zone contained the highest content of proteoglycans and had the lowest water content. The layer of calcified cartilage was a calcified zone that should have been clearly separated from the zone by a tidemark; in this case the tidemark was not visible. The collagen fibrils were arranged perpendicular to the articular surface and they penetrated from the deep zone through the calcified cartilage into the subchondral bone. As can be seen, the mineralized zone only contained a small volume of cells embedded in the calcified matrix; this part showed a very low metabolic activity (Fig. 3).

Microscopic evaluation of the repaired tissue within the defect site of the trochlea

The histological findings of the regenerated cartilage following the implantation of PHB/CHIT in the experimental group showed an overall good result of the newly formed repaired tissue. After six months of repair some of the sections from trochlear repaired tissue showed that the defect was repaired completely by hyaline cartilage. In these regions the microscopic appearance of the cartilage was normal cartilage, e.g. hyaline cartilage in which all the zones could be clearly identified. The regenerated cartilage displayed a good surface continuity showing a smooth intact articular surface. In some regions though, the surface was uneven and several layers even separated from the cartilage surface. Probably these layers represented parts of the synovial membrane. At higher magnifications, the chondrocytes in the superficial zone had a flat and elongated appearance and could be seen parallel to the surface. The result was intact, homogenous cartilage which exhibited a uniform stained extracellular matrix. There were no signs of hypocellularity and the cartilage were arranged into all the zones that was clearly visible and unaffected. In the deep zone the chondrocytes were arranged in vertical columns separated by a thin layer of extracellular matrix (Fig. 4).

Fig. 3. Histological section of articular cartilage, control group, stained with Alcian blue to demonstrate GAGs content

In another sample from the trochlea, the zonal arrangement could not be clearly observed, the surface showed good continuity but in the superficial layer the cells were small, elongated and the appearance of chondrocytes were lost. In the transitional layer cell proliferation was present; it was composed of large round chondrocytes that could be seen either singly or in pairs. In the deep part of the regenerated tissue the cells were more disorganised and they were not grouped into discrete columns (Fig. 5).

In one of the five animals, the trochlear sections showed that the cells were more disorganised and the zones could not be clearly observed (Graph 4). There were also clustering cells in the cartilage and the chondrocytes were not arranged into four zones; instead they formed these clusters that could be seen throughout the cartilage. Surface fibrillations were observed; this superficial fibrillation of the articular cartilage resulted in the loss of smoothness and it also showed signs of degeneration. Fissures that extended into both the superficial and transitional zones of the cartilage were observed. The affected cartilage demonstrated loss of Alcian blue staining reaching from the superficial zone into the transitional zone in which it was more manifested due to the proteoglycan loss. Changes could be observed in the superficial zone either as oblique splits or horizontal separation of the collagen bundles. This repaired tissue was of lower quality and differentiated from hyaline cartilage by its coarser fibrillary nature (Fig. 6).

Fig. 4. Microphotograph of articular cartilage, experimental group

- a) Microscopic appearance of the cartilage was normal and all zones clearly visible; b) + c) in some regions the surface was uneven and several layers were separated from the cartilage surface (arrows)

Fig. 5. Microphotograph of articular cartilage, experimental group

a) The defect was repaired with hyaline cartilage, which showed good surface continuity (arrow); b) cell proliferation was present (arrows), but zones were not clearly visible; c) chondrocytes were not grouped into a columnar organization

Fig. 6. Microphotograph of articular cartilage, experimental group

a) the defect was repaired incomplete, under the level of adjacent cartilage, in this zone cells were more disorganised; b) in some regions the cartilage showed decreased and uneven staining of proteoglycans (arrow); c) surface fibrillation; d) arrangement of chondrocytes in small clusters (arrow)

DISCUSSION

Histological analysis significantly contributes to both the assessment of cartilage damaged and the success of cartilage regeneration when evaluating cartilage repaired tissues and

its quality. R u t g e r s et al. reported that using more than one scoring system will give more information about the histological characteristics of the regenerated tissue [21].

A correlation can be seen between each three of these systems (O'Driscoll, Pineda, Wakitani) when it comes to

the cartilaginous repaired tissue and it suggests an overall good morphology for the regenerated cartilage. It was concluded by Moojen et al. that both O'Driscoll and Pineda scores were reliable semi-quantitative cartilage scoring systems with good correlation [11]. The values from all of the three scoring systems showed similar results even though the parameters and values were different; attention-getting differences between these three scores were the number of single criteria included. Pineda and Wakitani scores were classified as elementary systems since these systems only consists of 4 or 5 single criteria for evaluation, while the O'Driscoll score belonged to a complex system with 9 single criteria for evaluation. Orth and Dardry showed in a study which included the same three systems used in our work, that no significant disparity in reproducibility and reliability could be detected between the complex and elementary scores. Due to this study the authors suggested that these histological scoring systems represent a relatively potent tool for the evaluation of articular cartilage repair. However, it is important to note that the complex scoring systems will provide more descriptive information when it comes to the nature or character of the regenerated tissue, particularly about cellular and structural properties or the presence of degenerative changes [17]. Meaning that, for example O'Driscoll and Pineda, are validated scores which may increase the comparability of information and therefore stimulate consistency between the two studies.

During the histological analysis, there were a few characteristics of this repaired tissue noted, such as cluster formation, loss of matrix staining and some zones were not clearly visible; therefore it resulted in a lower score in comparison to the others.

Cartilage of lower quality, e.g. fibrocartilaginous repaired tissue may degenerate after a few years and does not tolerate mechanical loads over time, and it is connected with the risk of developing secondary osteoarthritis [2]. It is also important to note that hyaline cartilage repaired may still fail if mechanical irritation persists due to malalignment, poor defect fill or if the surface is disintegrated. Many characteristics are just as vital as the extracellular matrix composition when determining the quality of the repaired tissue. Examples of important characteristics are: cell organisations, viable cells with zonal specific arrangements, density of cells, surface integrity, cartilage-bone integration, and the orientation and organisation of the collagen fibrils [15].

The evaluation of the histologically stained sections provided a method to determine how closely the morphology and organisation of the repaired cartilaginous tissue resembles normal physiological articular cartilage and it is considered the gold standard for this purpose. For preclinical and clinical repair of cartilage, histological scoring provides a crucial outcome measure [5, 17].

Animal models in cartilage repair studies have been of a major importance for the development of effective treatments of cartilage injuries. For the introduction of new treatments and the use of them in clinical practice, *in vivo* animal studies are necessary for closing the gap between *in vitro* experiments and human clinical studies [6]. In the literature, it can be found that numerous large animal models may be used in cartilage repair studies. The ovine stifle joint defect model is widely accepted as an adequate model and are commonly used for the assessment of the efficacy of strategies for cartilage regeneration because of the anatomical similarity between their stifle joint and the human knee joint. Another advantage is the possibility to use arthroscopy for a "second look" which is considered beneficial. There are also limitations with the ovine model; the thickness of the articular cartilage in sheep is variable and can range between 0.4-1.6 mm [22]. This could lead to variable results within a study since this variability makes the volume of the defect created in the cartilage and subchondral bone likely to be different between individual animals. In other studies, the volume of cartilage and subchondral bone involvement has varied significantly and therefore the standard deviation between published studies are prominent. Smaller animals such as rodents or rabbits are generally accepted and used as a small animal model in initial lines of investigation. Nevertheless, in the final preclinical evaluation of a reconstruction technique for articular cartilage, often it requires a confirmation in large animal models [12]. At the moment, no perfect preclinical animal model exists but the authors concluded that the ovine is a readily accessible model for cartilage repair studies despite the previous mentioned limitations [1].

The terminal goal of all cartilage repair methods is to regenerate a repaired tissue with corresponding structure, biochemical and biomechanical characteristics of normal physiological hyaline cartilage. Studies have indicated that scientific advances in the area of tissue engineering have made important steps towards the development of normal articular cartilage repair. Especially, research has demon-

strated the potentiality of biomaterials physico-chemical character significantly influencing the differentiation, proliferation and matrix deposition by progenitor cells [8].

By filling an osteochondral defect with biomaterial, it may act as a template for the regeneration of new hyaline cartilage tissue. The idea is to use marrow stimulating technique such as subchondral drilling and implant biomaterial to guide and stimulate regeneration of the articular cartilage. In a review, P o t et al. concluded that on the whole, clinical studies showed improved cartilage regeneration when using biomaterials for implantation after bone marrow stimulation in animal models, but the authors also concluded that there is still room for enhancement regarding the clinical outcome and tissue quality [19]. Their conclusion is in line with this study in which we observed overall satisfactory results but also less satisfactory results in some aspects. Ideally, biomaterial should achieve regeneration by stimulating the recruitment of cells from the bone marrow and provide the biochemical and physical guidance that direct the cells to regenerate and built up the zones of articular cartilage to exhibit normal morphology [7].

A weakness of this study was the small number of animals making the number of samples available for scoring and evaluation limited. Also, not all parameters in the O'Driscoll score were used for grading in this study. The parameters bonding to the adjacent cartilage and freedom from degenerative changes in adjacent cartilage was not used in this evaluation since the samples only consisted of the defect. This might lead to an overestimation of the quality of the repaired tissue. To reach high objectivity in a study using histological scoring systems H o e m a n n et al. stated that two to three independent blinded readers is advisable to perform the evaluations [5]. To translate results from animal studies to human studies are always delicate matters; the cartilage in sheep is thinner compared to human cartilage and the created defects are only possible to make in the lower range of what is judged clinically significant for humans. For research purposes and further assessment of cartilage repaired tissues alternatives to histological scoring and analysis should be considered to get more information about the cartilage quality. Noticeable is also that translational values of animal models to clinical trials will depend on the comparability to the clinical situation, which in this case was the short follow up time. Many studies (also this one) investigated cartilage repaired tissues up to six months and this is a limiting factor for the

translational value since clinical improvements in humans often observed up to one and a half year after surgery [19].

Histological analysis of repaired tissues from large animal models can also be supplemented with analyses of the regenerated tissue biochemistry [5].

The overall results presented by histological scoring indicated that hyaline-like tissue was formed in most of the sheep. In one sheep out of five less satisfactory results were observed. The results suggest that a total score can indicate a level of regeneration and quality of the repaired tissues. Histological analysis remains the best method for scoring and describing the properties of the structure and morphological health of hyaline cartilage. Both complex scoring systems such as O'Driscoll and the elementary inverse systems of Pineda and Wakitani are well suited to describe the complex structures of animal cartilage repair.

CONCLUSIONS

In conclusion, the results of this animal study suggests that the scoring and histological findings demonstrated an overall successful *in vivo* repair and regeneration of articular cartilage. The discovered results provide further knowledge that could help improve the field of cartilage tissue engineering and regeneration associated with cartilage damage in an ovine animal model.

ACKNOWLEDGEMENT

This study was carried out within the framework of the VEGA project No. 1/0050/19 and APVV-17-0110.

REFERENCES

1. Ahern, B. J., Parvizi, J., Boston, R., Schaer, T. P., 2009: Pre-clinical animal models in single site cartilage defect testing: a systemic review. *Osteoarthritis Cartilage*, 17, 6, 705—713. DOI: 10.1016/j.joca.2008.11.008.
2. Armiento, A. R., Alini, M., Stoddart, M. J., 2019: Articular cartilage—Why does hyaline cartilage fail to repair? *Adv. Drug Deliv. Rev.*, 17 pp. DOI: 10.1016/j.addr.2018.12.015.
3. Berenbaum, F., Wallace, I. J., Lieberman, D. E., Felson, D. T., 2018: Modern-day environmental factors in the pathogenesis

- of osteoarthritis. *Nat. Rev. Rheumatol.*, 14, 11, 674—681. DOI: 10.1038/s41584-018-0073-x.
4. Bortoluzzi, A., Furini, F., Scire, C. A., 2018: Osteoarthritis and its management—Epidemiology, nutritional aspects and environmental factors. *Autoimmun. Rev.*, 17, 11, 1097—1104.
 5. Hoemann, C., Kandel, R., Roberts, S., Saris, D. B. F., Creemers, L., Mainil-Varlet, P., et al., 2011: International cartilage repair society (ICRS) recommended guidelines for histological endpoints for cartilage repair studies in animal models and clinical trials. *Cartilage*, 2, 2, 153—172. DOI: 10.1177/1947603510397535.
 6. Chu, C. R., Szczodry, M., Bruno, S., 2010: Animal models for cartilage regeneration and repair. *Tissue Eng. Part B Rev.*, 16, 1, 105—115. DOI: 10.1089/ten.TEB.2009.0452
 7. Levingstone, T. J., Ramesh, A., Brady, R. T., Brama, P. A., Kearney, C., Gleeson, J. P., O'Brien, F. J., 2016: Cell-free multi-layered collagen-based scaffolds demonstrate layer specific regeneration of functional osteochondral tissue in caprine joints. *Biomaterials*, 87, 69—81. DOI: 10.1016/j.biomaterials.2016.02.006.
 8. Matsiko, A., Levingstone, T. J., O'Brian, F. J., 2013: Advanced strategies for articular cartilage defect repair. *Materials*, 6, 637—668. DOI: 10.3390/ma6020637.
 9. Medvecky, L., Giretova, M., Stulajterova, R., 2014: Properties and *in vitro* characterization of polyhydroxybutyrate chitosan scaffolds prepared by modified precipitation method. *J. Mater. Sci. Mater. Med.*, 25, 3, 777—789. DOI: 10.1007/s10856-013-5105-0.
 10. Mescher, A. L., 2013: *Junqueira's Basic Histology: Text and Atlas*. 13th edn., New York: McGraw-Hill, 300—309.
 11. Moojen, D. J. Saris, D. B., Auw Yang, K. ., Dhert, W. J., Verbout, A. J., 2002: The correlation and reproducibility of histological scoring systems in cartilage repair. *Tissue Eng.*, 8, 4, 627—634. DOI: 10.1089/107632702760240544.
 12. Music, E., Futrega, K., Doran, M. R., 2018: Sheep as a model for evaluating mesenchymal stem/stromal cell (MSC)-based chondral defect repair. *Osteoarthritis Cartilage*, 26, 6, 730—740. DOI: 10.1016/j.joca.2018.03.006.
 13. Musumeci, G., Castrogiovanni, P., Leonardi, R., Trovato, F. M., Szychlińska, M. A., Di Giunta, A., et al., 2014: New perspectives for articular cartilage repair treatment through tissue engineering: A contemporary review. *World J. Orthop.*, 5, 2, 80—88. DOI: 10.5312/wjo.v5.i2.80.
 14. Musumeci, G., Castrogiovanni, P., Trovato, F. M., Di Giunta, A., Loreto, C., Castorina, S., 2013: Microscopic and macroscopic anatomical features in healthy and osteoarthritic knee cartilage. *OA Anatomy*, 1, 3, 30.
 15. Ng, H. Y., Alvin Lee, K. X., Shen, Y. F., 2017: Articular cartilage: Structure, composition, injuries and repair. *JSM Bone and Joint Dis.*, 1, 2, 1010.
 16. O'Driscoll, S., Keeley, F. W., Salter, R. B., 1986: The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. An experimental investigation in the rabbit. *J. Bone Joint Surg. Am.*, 68, 7, 1017—1035.
 17. Orth, P., Madry, H., 2015: Complex and elementary histological scoring systems for articular cartilage repair. *Histol. Histopathol.*, 30, 8, 911—919. DOI: 10.14670/HH-11-62.
 18. Pineda, S., Pollack, A., Stevenson, S., Goldberg, V., Caplan, A., 1992: A semiquantitative scale for histologic grading of articular cartilage. *Acta Anat. (Basel)*, 143, 4, 335—340.
 19. Pot, M. W., Gonzales, V. K., Buma, P., Int'Hout, J., van Kuppevelt, T. H., de Vries, R. B. M., Daamen, W. F., 2016: Improved cartilage regeneration by implantation of acellular biomaterials after bone marrow stimulation: a systematic review and meta-analysis of animal studies. *Peer J.*, 4, 2243—2269. DOI: 10.7717/peerj.2243.
 20. Ross, M. H., Pawlina, W., 2011: *Histology: A Text and Atlas*. Baltimore, Wolters Kluwer Health/Lippincott Williams & Wilkins, 198—207.
 21. Rutgers, M., van Pelt, M. J., Dhert, W. J., Creemers, L. B., Saris, D. B., 2010: Evaluation of histological scoring systems for tissue-engineered, repaired and osteoarthritic cartilage. *Osteoarthritis Cartilage*, 18, 1, 12—23. DOI: 10.1016/j.joca.2009.08.009.
 22. Sakata, R., Iwakura, T., Reddi, A. H., 2015: Regeneration of articular cartilage surface: morphogens, cells and extracellular matrix scaffolds. *Tissue Eng. Part B Rev.*, 21, 5, 461—473. DOI: 10.1089/ten.TEB.2014.0661.
 23. Tothova, C., Mihajlovicova, X., Novotny, J., Nagy, O., Giretova, M., Kresakova, L., et al., 2019: The serum protein profile and acute phase proteins in the postoperative period in sheep after induced articular cartilage defect. *Materials (Basel)*, 12, 1, 142. DOI: 10.3390/ma12010142.
 24. Wakitani, S., Goto, T., Pineda, S. J., Young, R. G., Mansour, J. M., Caplan, A. I., Goldberg, V. M., 1994: Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J. Bone Joint Surg. Am.*, 74, 4, 579—592.

Received August 16, 2019

Accepted September 19, 2019