Research Project Veterinary Medicine

The influence of microfracturing on fixation and degradation of a hydrogel in a horse model

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Summary

Articular cartilage lesions are a commonly encountered problem of the knee joint in humans. Cartilage has a limited ability to repair these lesions and the produced repair tissue is inferior to the original tissue. To overcome these limitations several surgical and non-surgical methods are developed to treat cartilage defects, including microfracturing and implantation of hydrogels. The current pilot study was performed to evaluate the influence of microfracturing on fixation and degradation of a hydrogel in the early stages after surgery in an equine model.

In the experiment 9 mature horses underwent surgery to create two full thickness cartilage defects with microfractures in one stifle and two full thickness cartilage defects without microfractures in the other stifle. In each stifle one defect was used to implant the hydrogel with fibrin glue to fixate the gel. In the other defect only fibrin glue was implanted to serve as a control. 3 different hydrogels were used in this study (hydrogel 1, hydrogel 2 and hydrogel 3). In three horses hydrogel 1 was implanted, in three horses hydrogel 2 was implanted and in three horses hydrogel 3 was implanted. After euthanasia at 1, 2 and 4 weeks post implantation, the harvested samples of the different defects were histological evaluated for 9 parameters (general presence of inflammatory cells, macrophages, neutrophils, eosinophils, collagen, capsule formation, blood vessel formation, presence of gel/fibrin glue and bone erosion).

The current study did not confirm a difference between microfracturing and no microfracturing on fixation and degradation of a hydrogel. However, this conclusion has to be taken with considerably caution. This study was designed as an pilot study to find major differences within the results and to use the information of this study to direct future full-scale research projects. The scope of this project was to obtain information and to make decisions towards the two techniques. Unfortunately, it was not possible to answer the research question with the gathered data. So additional research is recommended to investigate the influence of microfracturing on fixation and degradation of a hydrogel.

Contents

Introduction

Articular cartilage lesions are a commonly encountered problem of the knee joint in humans. Prevalence of cartilage lesions varies between 60% and 81% in Europe, based on the results of knee arthroscopies (Åroøen, 2004; Hjelle, 2002; Lewandrowski, 1997; Widuchowski, 2007). Several factors can be associated with the development of cartilage lesions, like direct trauma to the cartilage, joint injuries that alter normal biomechanics of the knee, developmental diseases, metabolic diseases or inflammatory diseases (Gersoff, 2000). Not only in humans but also in horses it is a major cause of lameness and associated with poor performance and early retirement (Cockelaere, 2016; Frisbie, 2006a; McCarthy, 2012; McIlwraith, 2016), but no studies about the prevalence of cartilage defects in horses are available.

Cartilage has a limited ability to repair these lesions and the produced repair tissue is inferior to the original tissue. The formed fibrocartilage with collagen type I has limited structural and function properties compared to the original collagen type II containing hyaline cartilage. It has a shorter longevity and it does not integrate well with the surrounding hyaline cartilage (see next section for more details about cartilage repair) (McCarthy, 2012; Cockelaere, 2016). To overcome these limitations research has focused and is still focused on restoration of joint function, pain relief, prevention or delay of the onset of osteoarthritis and regeneration of cartilage. Several surgical and non-surgical methods are developed to treat cartilage defects. In the experiment described in this paper two of these treatment options are used, hydrogels and the microfracture technique. A lot of research has already been done on these two techniques, but most are focused on the long-term effects. There is little to no information about the early effects of both methods. Therefore, the aim of this pilot study is to evaluate the influence of microfracturing on fixation and degradation of a hydrogel in the early stages after surgery. In the experiment nine horses underwent surgery to create two full thickness cartilage defects with microfractures in one stifle and two full thickness cartilage defects without microfractures in the other stifle. In each stifle one defect was used to implant the hydrogel, the other defect is used as a control. After euthanasia at 1, 2 and 4 weeks post implantation, the harvested samples of the different defects were histological evaluated.

The equine model was chosen for various reasons. A study comparing cartilage thickness in humans and several animal species showed cartilage thickness in the stifle of a horse is most comparable to cartilage thickness in the human knee. Beside this, it has a similar organization of glycosaminoglycans, collagen and DNA as human cartilage (Frisbie, 2006b; Malda, 2012). As mentioned before cartilage lesions are not only a problem in humans, they are also a commonly encountered problem horses. This makes the use of an equine model also useful for equine veterinary medicine. A more practical reason is the fact that it is more easy to perform surgery on larger animals.

In the next section some general aspects of cartilage structure, cartilage damage, repair and possible treatments will be discussed.

General aspects

Articular cartilage composition, structure and function

A joint is a multifunctional part of the body because it has to satisfy very different demands. It has to be as rigid as the connected bones, because the forces generated during exercise are transmitted from bone to bone through the joint. It provides smooth motion of the articulating bony ends and it absorbs the shock produced during locomotion. Articular cartilage is the structure in a joint that is necessary to accomplish these functions (McIlwraith, 2016). Articular cartilage is a subtype of hyaline cartilage, also present in ribs, nose, larynx, bronchus and trachea (Jung, 2014). Articular cartilage provides a lubricated surface for movable joints and it provides load transmission and distribution over the joint, because of its special conformation. It is composed of four different layers based on the structure of the extracellular matrix and cell morphology, the *superficial layer*, *middle layer*, deep layer and *calcified cartilage layer* (figure 1) (Sophia-Fox, 2009).

The function of the *superficial layer* is to provide lubrication and to ensure smoothness during movement of the joints. It contains flattened chondrocytes and articular chondroprogenitor cells (APCs), a small amount of proteoglycans, a large amount of water and a dense network of collagen type II fibrils parallel to the articular surface. The *middle layer* functions as transition between the shearing forces on the superficial layers and compressing forces in the lower layers. It has a higher amount of proteoglycans and a lesser amount of water compared to the superficial layer. The chondrocytes here are round instead of flattened. The collagen fiber network is less dense compared to the superficial layer and is randomly organized. Finally, the *deep layer* has the function to provide resistance to compression and to distribute loads. In this layer the chondrocytes are arranged in columns perpendicular to the articular surface. It has the lowest amount of water and the highest amount of proteoglycans. It also has the highest amount of collagen fibrils perpendicular to the articular surface. These three layers together form the hyaline cartilage. A so-called 'tidemark' separates the third layer from the fourth layer. This is the *calcified cartilage layer*, which is responsible for the anchorage of the resilient cartilage to the rigid bone (Jung, 2014; McIlwraith, 2016).

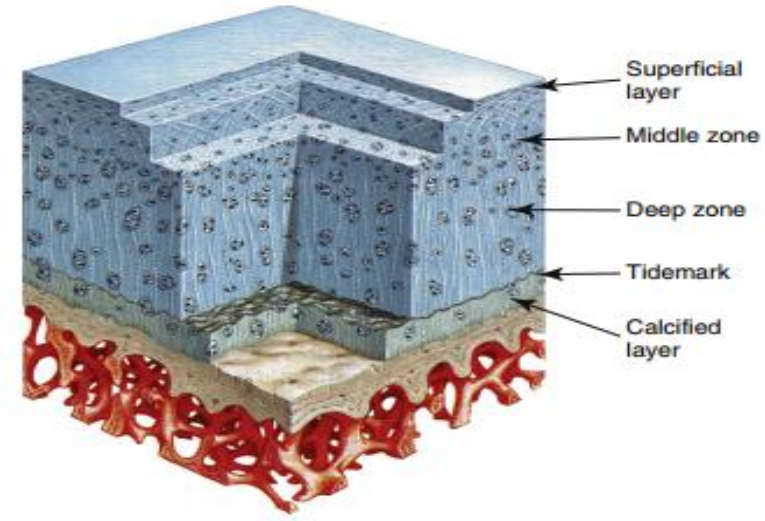

Figure 1: Schematic image of the different layers of articular cartilage, sitting on the compact subchondral bone and the underlying trabecular bone (McIlwraith, 2016).

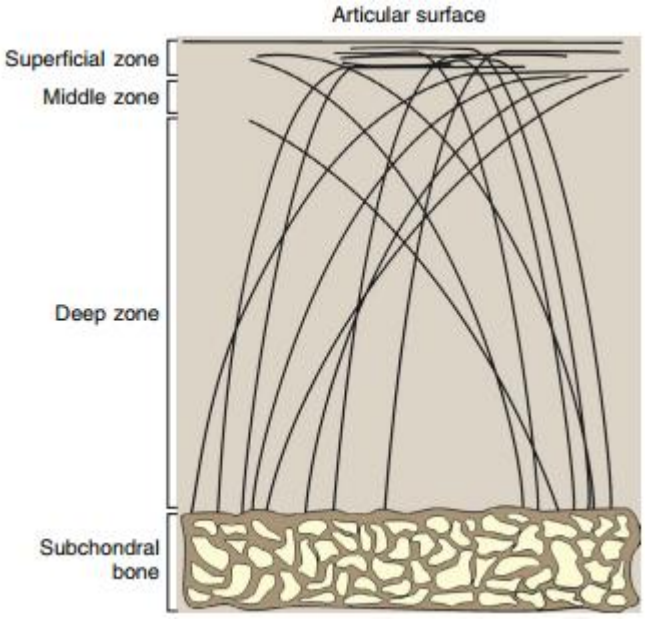

Figure 2: Schematic overview of the configuration of the collagen network in articular cartilage, forming an arcade-like structure (McIlwraith, 2016).

Articular cartilage consists of chondrocytes (1% - 5%), type II collagen (12% - 21%) and extracellular matrix. Cartilage formation starts with the differentiation of mesenchymal stem cells, also known as mesenchymal stromal cells (MSCs), which come from the bone marrow to turn into round *chondroblasts*. These chondroblasts actively form hyaline cartilage, but only in embryonic joints and immature animals (Gill, 2006). Production of the extracellular matrix traps each chondroblast within this matrix, thereby separating chondroblasts from each other. Each separated chondroblast undergoes one or two mitotic divisions to form a mature cell, known as a *chondrocyte*, which then loses its ability to produce cartilage matrix. In adult cartilage each chondrocyte is located in a space called *lacuna*. Chondrocytes are responsible for the maintenance of the matrix, but they are not able to actively produce its constituents (Young, 2000). Beside chondrocytes also *articular chondroprogenitor cells* (APCs) are found in the superficial cartilage layer (Dowthwaite, 2004). These cells have shown *in vitro* the capacity to differentiate in either chondrogenic, osteogenic or adipogenic cells, similary to MSCs. MSCs differentiated into chondrocytes produce a matrix composed by collagen of type II and type X; this is typically seen in hypertrophic chondrocytes during endochondral ossification. On the other side, APCs have shown to produce only type II collagen, suggesting that these cells when differentiating will be able to form hyaline cartilage and may prove to have superior potential for cartilage repair (McCarthy, 2012).

The solid component of cartilage is composed mainly by *type II collagen*. Collagen is constituted by flexible fibers with a high tensile strength and low shearing strength, which enables the tissue to adapt to changes and movements (Eurell, 2006). Collagen is secreted into the extracellular matrix (ECM) as *tropocollagen*, a molecule with three polypeptide chains. In the extracellular matrix the tropocollagen molecules polymerise to collagen fibrils, and subsequently bundles of these fibrils together form a collagen fiber (Young, 2000). As described earlier the fibers are differently orientated in the different layers. Together the network of fibrils forms an arcade-like structure with proteoglycan aggregates trapped within it (figure 2) (McIlwraith, 2016).

The ECM where the chondrocytes rest is composed of water (65% - 80%), proteoglycans (6% - 10%), glycoproteins and lipids (2% - 3,5%) (Sophia-Fox, 2009). *Proteoglycans* are macromolecules with a protein core and glycosaminoglycan carbohydrate tails connected to it. These molecules are anchored to the collagen fibrils either directly or via hyaluronic acid molecules. *Aggrecan* is the largest and most abundant proteoglycan in the ECM and it is composed of a protein core with several hundreds of keratan sulfate and chondroitin sulfate side chains. The aggrecan monomers are attached to each other via hyaluronic acid and link proteins, forming larger aggregates. The sulfate groups of both side chains are negatively charged and can attract water, leading to swelling of the ECM. However, this swelling is limited, because the surrounding collagen network is not elastic and for this reason, it will not expand (McIlwraith, 2016). In this complex structure a balance is generated by the osmotic pressure of the proteoglycan aggregates and the restraint of the collagen fibers, resulting in a specific pressure within the cartilage. This balance gives cartilage its unique viscoelastic properties. When external pressure is exerted on the cartilage the water will be squeezed out, while when the external pressure is diminished or released the water will be drawn back into the ECM. This mechanism enables cartilage to withstand compressive loads and provides elasticity to the tissue (McIlwraith, 2016). This process is also important for delivery of nutrients to the cartilage. Because cartilage has no blood vessels, it needs another way to obtain sufficient nutrients. The alternate pressure and release of cartilage tissue during exercise works as a pump pushing fluid out and

drawing it back in. When the fluid flows out it is mixed with the surrounding synovial fluid which contains nutrients. This nutrient rich mix is then drawn back in to the cartilage. This type of diffusion enables cartilage to survive without the presence of blood vessels (McIlwraith, 2016).

Cartilage damage and repair techniques

Damage to cartilage can arise due to trauma or to degeneration of the tissue. Articular cartilage has no blood vessels, lymph vessels or nerves. The absence of nerves means that mild damage to the cartilage will not be detected by the body. It will gradually progress over time and become clinically evident when the damage involves the surrounding structures (McIlwraith, 2016).

Cartilage injury leads to disruption of the collagen network and damage to the cells. When the collagen network is disrupted, a secondary loss of proteoglycans will occur, because they are no longer trapped within the network. Cell damage results in apoptosis, necrosis and enzymatic degradation of collagen and proteoglycans, either directy or via cytokines. An inflammatory response is triggered in the synovium by disruption of the extracellular matrix and release of its contents into the joint. Subsequently repair of the lesion starts, with limited cell replication, increased synthesis of extracellular matrix and reorganization of the matrix by endogenous cells (Gill, 2006). Although some APCS are found in the superficial cartilage layer, the intrinsic ability for healing and repair of the damage is limited (Dowthwaite, 2004; Gill, 2006). If left untreated, the damage will worsen and eventually lead to osteoarthritis (Emans, 2014).

Because of the limited ability for self-repair, several therapies have been developed to improve healing of cartilage damage. These therapies may be either conservative or surgical. Conservative treatments involves primarily the use of different drugs treatment, such as non-steroidal anti-inflammatory drugs, glucosamines, cortisone or hyaluronic acid. The main goal of these therapies is to provide pain relief and reduce inflammation. Physiotherapy is also widely used to address joint diseases, improve joint function and reduce pain. Conservative treatments, however, have no influence on cartilage repair, but are used to slow down progressive degeneration and to reduce symptoms such as pain (Erggelet, 2008).

Surgical therapies can be divided in three different categories, they can be either palliative, reparative or restorative.

Palliative

Palliative techniques include debridement and lavage. With debridement of full-thickness lesions loose cartilage fragments are removed to promote intrinsic repair and to reduce shedding of debris into the synovial environment (Cokelaere, 2016). The best results are obtained when the calcified cartilage layer is removed, but the underlying subchondral bone is left intact (Frisbie, 2006a). Lavage of the joint is performed after debridement to remove detached cartilage components, like collagen fibrils and proteoglycans, which may act as inflammatory mediators.

This technique relieves pain, but is symptomatic and effective only temporarily effective. It results in the formation of fibrocartilage and imperfect hyaline repair tissue. The fibrocartilage produced in this case is functionally inferior to the original hyaline cartilage, because it has a shorter longevity and does not integrate well with the surrounding tissue (Erggelet, 2008; Cokelaere, 2016).

Reparative

Reparative techniques stimulate the bone marrow by exposure of the underlying subchondral bone. As a result bleeding occurs into the defect, supplying a small amount of MSCs and growth factors to stimulate repair (Frisbie, 1999). Three different methods have been used in clinics: abrasion arthroplasty, drilling and microfracture. With *abrasion arthroplasty* the damaged cartilage and underlying calcified cartilage are removed to expose the subchondral bone. A round or oval burr is used to abrade the subchondral bone until the whole surface contains punctuate bleeding bone. With *subchondral drilling* the cartilage and the superficial bone are removed with small trephines. Multiple drill holes are created that penetrate into the subchondral bone. The *microfracture* technique is similar. An handheld awl is used to perform small penetrating fractures into the subchondral plate. This technique allows more precision and avoids possible thermal damage that may occur when using a drill (Brittberg, 2011). Advantages of microfracture compared to the other two techniques are the reduced thermal damage and the production of a more rougher surface to which repair tissue might adhere more easily (Smith, 2005). Furthermore, the holes made with the microfracture technique are smaller (0,5 – 1 mm in diameter) compared to, for example, the holes made with drilling (2,0 – 2,5 mm in diameter), causing less alteration of the biomechanics of the subchondral bone plate (Hunziker, 2002).

Restorative

Restorative techniques attempt to replace damage cartilage with hyaline cartilage or hyaline-like tissue instead of fibrocartilage. *Autologous chondrocyte implantation* (ACI) is a restorative technique where a full thickness cartilage sample is taken from a non-weight bearing region of a joint. The chondrocytes from this biopsy are then cultured *in vitro* and subsequently implanted in the cartilage defect of the same patient and covered with a periosteal flap. This requires a two-step procedure, making it a laborious technique. Also it requires a long recovery and implies a risk for donor site morbidity. However, the advantage is the use of patient's own material to prevent rejection of the body (Makris, 2015). *Mosaïc arthroplasty* is restorative technique where one or more cylindrical osteochondral transplants are taken out of a non-weight bearing region of the joint. These transplants are transferred to the defect to create a new intact cartilage surface. A larger biopsy is necessary, in this case, with a higher risk for complications of the donor site compared to ACI, however, with this technique only one surgery is necessary (Erggelet, 2008).

Novel approaches

None of the techniques described above results in formation of hyaline cartilage that is functionally equal to the original tissue, so the long-term prognosis is still uncertain. For this reason novel tissue engineering strategies are being explored in an attempt to overcome these limitations. The use of hydrogels is one of these strategies (Cokelaere, 2016). Hydrogels are three-dimensional, hydrophilic, polymer networks swollen in water, made of natural-derived or synthetic material. Their structure is intended to be comparable with cartilage, a network of proteoglycan polymers and collagen swollen in water, to obtain similar swelling and lubricating properties (Spiller, 2011). Hydrogels are used as cell-free of cell-laden scaffolds. Cell-free scaffolds are used for efficient load transfer after implantation and to recruit cells for cartilage repair. Based on their composition hydrogels can mimic the ECM and in this way promote tissue growth. Cell-laden scaffolds act as a carrier of different

types of cells to stimulate the body to produce cartilage (Vega, 2017). However, it is difficult to create a hydrogel that integrates well with the surrounding tissue. A joint surface is a complex structure and chondrocytes have a limited ability for repair. This results in formation of a discontinuous surface between the implant and the surrounding cartilage leading to formation of new defects at the periphery and eventually failure of the implant. Research is focused on improving integration of hydrogels, but no permanently implantable hydrogels are available yet (Spiller, 2011).

In this study three different types of hydrogel are used in combination with and without the microfracture technique to assess fixation of the hydrogels and repair of the cartilage defects.

Cartilage repair with and without microfractures

Chondral lesions are described as partial thickness or full thickness defects. A partial thickness lesion is a defect in the superficial zone and sometimes extending into the middle zone. Full thickness lesions extend through all cartilage layers, but leaves the subchondral bone intact. Osteochondral lesions are defects extending through all the cartilage layers and penetrating the underlying subchondral bone (Angel, 2003).

The aim of the microfracture technique is to stimulate cartilage repair mediated by the bone marrow, in order to obtain optimal results. To perform this technique it is necessary to remove the calcified cartilage while leaving the underlying subchondral bone intact. Subsequently, several holes are made by an handheld awl or pick perpendiculary to the articular surface. The holes should be 3 to 5 mm apart from each other and covering the entire debrided area. They need to be about 2 to 4 mm deep to allow good access to the bone marrow (Cokelaere, 2016). Granting access to the bone marrow leads to a response that can be divided in three phases. The *first phase* is an immediate response with apoptosis of damaged cells, loss of glycosaminoglycans, rupture of collagen, cartilage swelling and hemarthrosis (Angel, 2003; Lotz, 2010). The *second phase* starts when a blood clot has formed in the defect. During the second phase loss of glycosaminoglycans and matrix degradation continues. The blood clot contains various constituents, including platelets, undifferentiated bone marrow cells and blood cells. This platelets present here release several cytokines and growth factors leading to infiltration of leucocytes. Meanwhile, the blood clot gradually forms a dense fibrous network with the inflammatory cells and undifferentiated bone marrow cells within it (Angel, 2003; Hunziker 2002; Olsen, 2015). The third phase is the real repair phase. This starts when the fibrinous mass in the defect is infiltrated by small, new blood vessels. The undifferentiated cells differentiate into fibroblasts and start to produce granulation tissue, which undergoes progressive hyalinization and chondrification forming a fibrocartilagenous matrix that fills the defect (Gill, 2006).

Without the use of the microfracture technique the repair mechanism is slightly different. In this case only the cartilage is damaged while the underlying subchondral bone remains intact. As a result inflammatory cells and undifferentiated cells from the bone marrow are not present for the repair of the defect. Chondrocytes are capable to initiate the repair, however they are trapped within their lacunae and not able to migrate through the matrix to reach the defect. Thus, only the chondrocytes at the site of injury are available. Furthermore, the ability of chondrocytes to proliferate decreases when the skeleton matures, making it impossible for the chondrocytes to completely repair the defect (Angel, 2003). However, also defects without access to the bone marrow can fill with repair tissue. A possible explanation for this observation may be given by the presence of mesenchymal

progenitor cells in the synovium. The synovium lines the inner surface of a synovial joint capsule. Synovium is a specialized collagenous tissue responsible for the secretion of synovial fluid for the lubrication of articular surfaces (Young, 2000). Several cell types are found in the synovium. Type A synoviocytes are tissue macrophages with the ability to actively phagocytose cell debris and waste in the joint space. Type B synoviocytes are fibroblast-like cells producing synovial fluid and matrix constituents like hyaluronan, collagen and fibronectin (Iwanaga, 2000). Synovium also contains mesenchymal progenitor cells and it has been postulated that they possibly play a role in repair of cartilage defects without bone marrow involvement. Several studies showed the chondrogenic potential of these cells when cultured *in vitro* (Chen, 2016; De Bari, 2001; Fickert, 2003; Jo, 2007; Matsumura, 2017). Also *in vivo* studies showed the presence of mesenchymal progenitor cells in the synovium of the knee joint and the ability of these cells to repair full thickness cartilage defects (Koga, 2008; Kurth, 2011; Lee, 2012; Miyamoto, 2007).

However the main problem in cartilage repair is the formation of cartilage with an inferior quality compared to the native tissue. Cartilage with some hyaline characteristics, like collagen type II, can be formed in the early repair phase. However, later on in remodeling the amount of collagen type I increases and over time (6 – 12 months) the repair tissue gradually changes into fibrocartilage (Angell, 2003). This fibrocartilage is functionally incomparable to the original hyaline cartilage, because it has a shorter longevity and does not integrate well with the surrounding hyaline cartilage (Erggelet, 2008; Cokelaere, 2016). When fibrocartilage is formed in a defect or a defect is not completely filled with repair tissue an abnormal cartilage surface is obtained. As a consequence, the biomechanical functionality cannot be maintained, resulting in a decreased capacity to withstand loading. This will eventually lead to new damage, creating a circle of overloading and damage to the new fibrocartilage tissue, eventually resulting in the development of osteoarthritis (McIlwraith, 2016).

Cellular response in cartilage repair: the foreign body reaction

Inflammation is a general response of the body to trauma. This process will start after creating the defects and microfractures, triggered by the damage caused to the tissue. The body may respond with a non-infectious inflammation when biomaterials, such as hydrogels, are implanted to promote cartilage repair. This reaction is called the ‘foreign body reaction’ and consist of four stages, which are acute inflammation, chronic inflammation, formation of granulation tissue and eventually fibrous encapsulation (Jones, 2007; Ratner, 2013).

When a biomaterial is placed *in vivo*, the surrounding tissue gets injured and this injury leads to an acute inflammatory response. Neutrophils are the first cells present, but live only for about 24 to 48 hours. They are responsible for phagocytosis of foreign material and microorganisms, are involved in debridement of the injured tissue and produce several macrophage chemotactic molecules to attract macrophages to the site of injury (Bastian, 2011; Ratner, 2013).

Macrophages are the most important cells in the foreign body reaction. They are recruited after the neutrophils and continue trying to phagocytose the material and secrete enzymes to digest it. The process of phagocytosis of a foreign material starts with recognition of the material. Subsequently the cells attach to it, followed by engulfment and degradation. Biomaterials are not always recognized as ‘foreign material’. If a material is recognized as foreign the degree of engulfment and

degradation depends on the type and size of the material. In general phagocytosis of biomaterials occurs succeeds when the material is less than 5 µm and fails when it is larger than 5 µm. The inability of cells to phagocytose the material may lead to a process called 'frustrated phagocytosis', a process during which several enzymes are released by the surrounding cells in an attempt to degrade the material. Instead of intracellular lysis of engulfed material extracellular proteases, toxic oxygen metabolites and cytokines are released by neutrophils and macrophages. Fusion of multiple macrophages leads to the formation of one giant multinucleated cell. During a foreign body reaction, giant cells are not always formed as this depends on the type and form of the material, and they are seen more commonly when foreign body display a rough surface (Ratner, 2013).

The intensity and duration of the inflammatory response depends on the type of biomaterial used. Usually acute inflammation resolves after about one week. A longer period of acute inflammation, associated with the presence of neutrophils, could indicate the presence of an infection. After implantation of a biomaterial macrophages continue trying to degrade the material through phagocytosis and secrete enzymes. As a result, acute inflammation progresses into chronic inflammation (Yu, 2015). This process can start within a few days after injury and may last for weeks, months or even years if inflammatory stimulus remains present. The biomaterial itself can trigger an inflammatory response, but also motion of material or an infection can lead to chronic inflammation. In most cases a chronic inflammatory site contains macrophages, monocytes, lymphocytes and plasma cells. However, in the foreign body reaction macrophages are the predominant cells. Lymphocytes and plasma cells are part of the adaptive immune system. They are primarily involved in immune reactions, stimulate antibody production and may trigger hypersensitivity reactions. These cells are capable to recognize MHC (major histocompatibility complex) proteins on cell surfaces of the body's own cells and unknown cells, in this way enabling it to differentiate between self and non-self. For example, in the case of organ or cell transplantation the cell or organ is recognized as non-self by the adaptive immunity leading to antibody production and eventually rejection. In contrasts to organ or cell transplantations, implantation of a biomaterial without viable cells does not result in rejection. Biomaterial implants lack the MHC proteins and as a result of are not recognized by the adaptive immune system (lymphocytes) as non-self and no antibody production occurs. Sometimes lymphocytes are present in nonimmunologic reactions or inflammation, but their role in these situations is not completely understood (Jones, 2007; Ratner, 2013).

Formation of granulation tissue is a normal process in wound healing after biomaterial implantation. Active fibroblasts proliferate, producing collagen and proteoglycans, and new blood vessels are formed. This can start as early as 3 -5 days after injury (Jones, 2007). The newly formed granulation tissue is the precursor for fibrous encapsulation. The fibrous capsule consists of collagen fibers and fibroblasts and forms when neutrophils and macrophages fail to phagocytose the material. It is the final attempt of the body's innate immune system to separate the material from the rest of the body (Anderson, 2008; Ratner, 2013). Human *in vitro* studies show capsule formation at 4 to 6 weeks (Saha, 2013; Yang, 2013). *In vivo* studies with rabbits and monkeys shows capsule formation around 4 weeks (Ravanetti, 2015; Powers, 1986; Stone,1997).

Hypothesis

The hypothesis is that the microfracture technique stimulates healing of an cartilage defect by new blood supply and an inflammatory response resulting in better filling with repair tissue and a better fixation of a hydrogel compared to a defect without a microfracture. But it is also suspected that microfracturing leads to an increased degradation of a hydrogel, as a result of the presence of phagocytic inflammatory cells, like neutrophils and macrophages.

Materials & Methods

Nine clinically healthy, mature Pinto horses were used, both mares and geldings. All animals were subjected to a general health examination, followed by a specific evaluation to exclude the presence of lameness and joint effusion. Furthermore, a radiological examination was performed to exclude any pre-existing conditions.

Surgical procedure

The horses were fasted 7 to 8 hours before surgery. On the day of surgery a preoperative blood sample (serum) was taken from each horse. This blood sample was used to detect problems that cannot be recognized with the physical examination and are import for the anesthesia procedure, for example liver, kidney and muscle values. The horses received prophylactic penicillin (20 mg/kg IM) and gentamycin (6.6 mg/kg IV) and were sedated with acepromazine (0,025 mg/kg IM) and xylazine (1,1 mg/kg IV). Phenylbutazone was administered orally to relief pain (4,4 mg/kg). Induction of the horses was achieved through the administration of diazepam (0,05 mg/kg IV), ketamine (2,2 mg/kg IV) and lidocaine (2,0 mg/kg IV). General anesthesia was maintained with isoflurane inhalation.

During surgery the horses were placed in dorsal recumbency and an incision of 5 – 6 cm was made in the left and right stifle, medial from the middle patellar ligament to gain access to the left and right femuro-patellar-tibial joint space.

In both stifles two defects were made in the medial femoral trochlear ridge. The performed procedure for one stifle will be described first. Two defects with diameter of 6 mm and full cartilage thickness were created with a biopsy punch, hand drills and curettes. Subsequently several microfractures were made in both defects using a handheld awl. This was followed by flushing the joint. A hydrogel was implanted in one of the two defects, with fibrin glue to fixate the gel in the defect. In the other defect only fibrin glue was implanted to serve as a control. After implantion of the hydrogel and the fibrin glue the joint capsule and subsequently the fascia were closed with a simple interrupted suture with Vicryl 0. The subcutis was closed with Vicryl 2/0 in a continuous suture pattern and the skin with a continuous suture with Nylon 0. A stent was placed over the wound and sutured with Nylon 0. For the other stifle the followed procedure is similar as described above. Again two defects with diameter of 6 mm and full cartilage thickness were created in the medial femoral trochlear ridge. However, in these two defects no microfractures were made. After flushing of the joint the same hydrogel as used in the other stifle was implanted with fibrin glue in one of the two defects. Again only fibrin glue was implanted in the other defect to serve as a control. Closure of the wounds is similar as described for the other stifle.

In this experiment three different types of hydrogel were used. These hydrogels were developed by the University of Utrecht (gel 1), the University of Dresden (gel 2) and the University of Würzburg (gel 3). It is beyond the scope of this paper to discuss in detail the chemical composition and properties of these hydrogels. In three horses hydrogel 1 was implanted, in three horses hydrogel 2 was implanted and in three horses hydrogel 3 was implanted. With the use of a randomization software it was randomly assigned which horse received which type of hydrogel. This same software was used to assign to each animal in which stifle the microfractures were made and if the hydrogel was implanted in the proximal or distal defect. In table 1 the disposition for horse 1 is shown as an example.

	Left stifle	Right stifle
Proximal defect	Gel 3	Gel 3 (microfracture)
Distal defect	Fibrin glue	Fibrin glue (microfracture)

Table 1 - This figure shows the disposition for horse 1. In this horse the gel was implanted in the proximal defects of both stifles and the fibrin glue in the distal defects. Microfractures were made in both defects of the right stifle.

Each day surgery was performed on 3 horses, so three consecutive days were necessary for the surgery of the 9 horses. Each horse on the same day of surgery received another type of hydrogel (see table 2).

Day of surgery	Horse	Type of hydrogel	Euthanasia (days after surgery)
1	6	Hydrogel 1	7
	5	Hydrogel 2	7
	7	Hydrogel 3	7
2	4	Hydrogel 1	14
	3	Hydrogel 2	14
	1	Hydrogel 3	14
3	2	Hydrogel 1	28
	9	Hydrogel 2	28
	8	Hydrogel 3	28

Table 2 – Schematic overview of the disposition of the horses (column 2) at the different surgery days (column 1). On each day of surgery hydrogel 1, 2 and 3 were represented (column 3). The fourth column shows how many days after surgery euthanasia was performed. Also at these three different time points (7, 14 and 28 days) hydrogel 1, 2 and 3 were represented.

After surgery all animals were put on box rest. Gentamycin (6,6 mg/kg IV once daily) and penicillin (20 mg/kg IM or IV once daily) were administered for 8 days. Pain management consisted of oral administration of meloxicam (0,6 mg/kg) for 14 days. The stents were removed 2 days after surgery. Every horse was assessed daily for their overall health, weight bearing, signs of joint effusion and signs of a possible wound infection.

Sample collection

Three different times points were chosen for comparison of the three materials (see table 2). To this purpose, the first 3 horses who had undergone surgery were euthanized 7 days post-operatively. Each horse having another type of hydrogel implanted (see table 2). The second 3 horses who had undergone surgery were euthanized 14 days post-operatively. Again each horse had a different hydrogel implanted. The final 3 horses were euthanized 28 days post-operatively.

After euthanasia samples of the lesions in the medial femoral trochlear ridge were taken out of the cartilage and underlying subchondral bone as a square block with a diameter of approximately 1 cm by our colleagues of the Veterinary Faculty at the National University in Costa Rica. The samples were labeled and fixated in formalin before transportation to the Veterinary Faculty in Utrecht, the Netherlands, for histological examination.

Sample preparation

After arrival at the Veterinary Faculty in Utrecht the samples were cut in half. Half of the sample were placed in a Luthra solution for decalcification, the other half were placed in an EDTA solution. The Luthra solution is mixture of 800 ml distilled water, 100 ml 37% hydrochloric acid and 100 ml 100% formic acid. After six days of decalcification the samples were processed by an automatic tissue processor (see appendix I). Subsequently embedding of the samples was performed by placing the samples in the middle of a cassette filled with molten paraffin wax. After cooling down the cassettes were removed so the samples were ready for section cutting with a microtome.

Histological and statistical evaluation

For the histological evaluation the samples were stained with Hematoxylin and Eosin (H&E) (see appendix II). All samples were evaluated for 9 different parameters to assess the fixation and degradation of the hydrogels:

- Presence of inflammatory cells
    - Specific presence of macrophages
    - Specific presence of neutrophils
    - Specific presence of eosinophils
- Presence of collagen formation
- Presence of capsule formation
- Presence of blood vessel formation
- Presence of gel/fibrin glue
- Presence of bone erosion

Each parameters was scored at a scale from 0 to 3

- 0 = not present
- 1 = small amount present
- 2 = moderate amount present
- 3 = large amount present

Based on these results it was not possible to do a statistical analysis, because of a lack of sufficient data. To solve this problem the data was converted into binary data. The previous scores of 0 (not present) and 1 (small amount present) were taken together as one group and the previous scores of 2 (moderate amount present) and 3 (large amount present) were taken together as one group. Subsequently an logistic regression analysis with random horse effects was performed.

Results

Of each sample several slides were made for histological assessment with a microscope. All samples contained more or less repair tissue in the created defects (see figure 4). In 10 of the 36 samples a part of the tissue was lost during processing, which impaired the evaluation (see figure 3). All samples were evaluated for nine parameters:

- General presence of inflammatory cells
- Specific presence of macrophages
- Specific presence of neutrophils
- Specific presence of eosinophils
- Presence of collagen
- Presence of capsule formation
- Presence of blood vessels
- Presence of gel/fibrin glue
- Presence of bone erosion

Each parameter was scored at a scale of 0 – 3 (0 = not present, 1 = little amount present, 2 = moderate amount present and 3 = large amount present). See appendix III for examples of sample scoring. The results are represented in table 1 (see appendix IV).

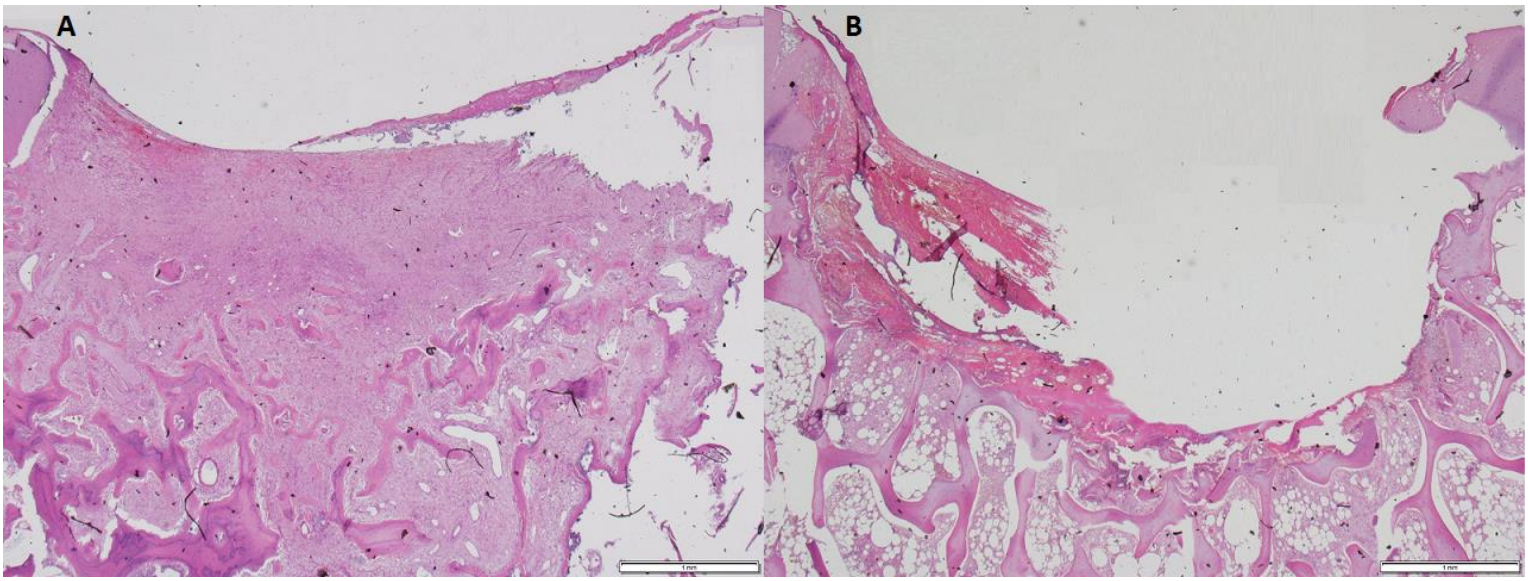

Figure 3: Examples of slides with partially lost tissue during processing
A: histological slide missing the right part of the defect (gel 1 with microfracture, week 4), B: histological slide missing almost all the repair tissue (gel 2 without microfracture, week 1).

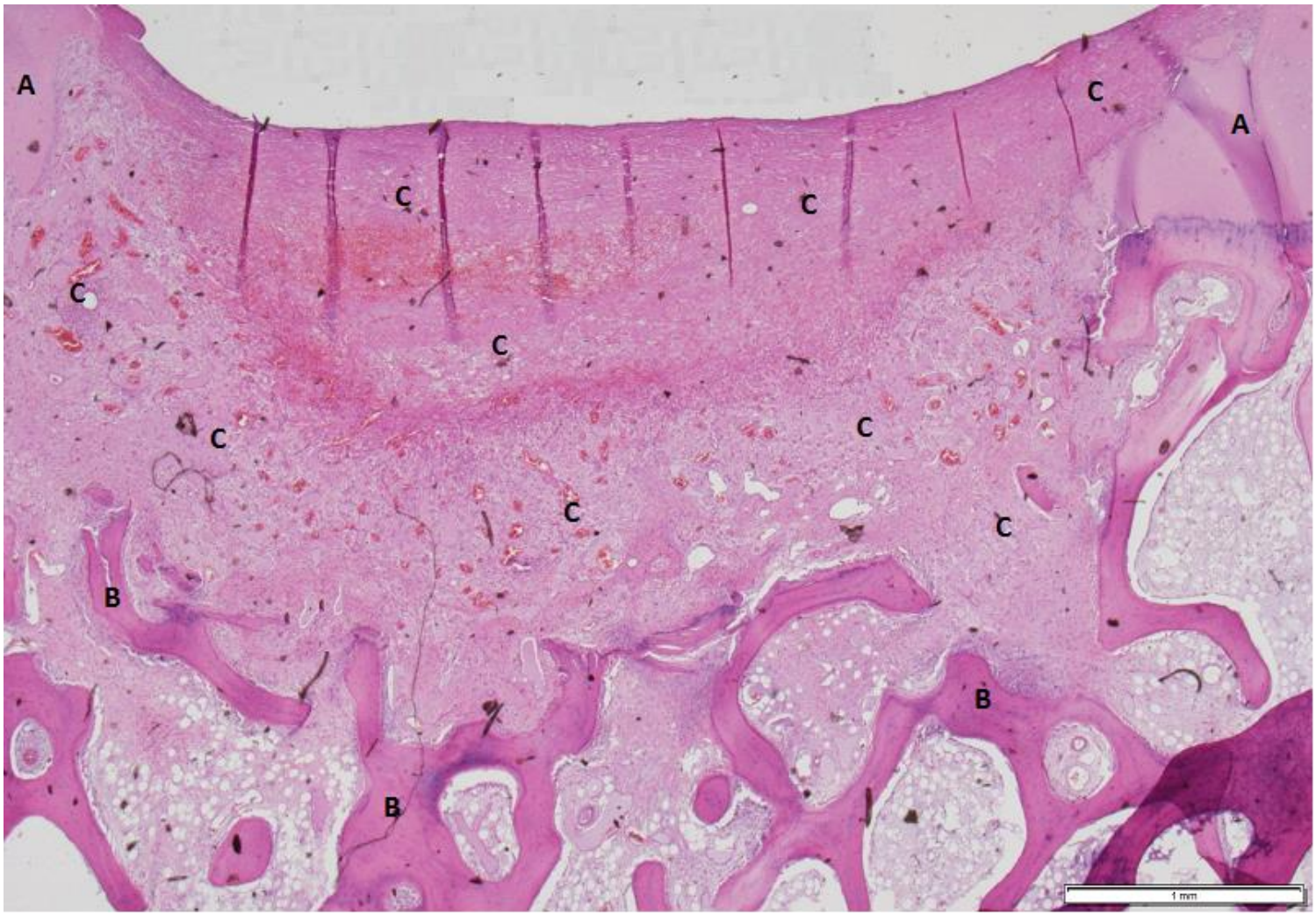

Figure 4: Example of an histological slide (control 1 without microfracture, week 2)
A: cartilage, B: Bone, C: repair tissue filling the defect

Statistical analysis

Based on the results as represented in table 1 (appendix IV) it was not possible to do a statistical analysis because of a lack of sufficient data. To solve this problem the data was converted into binary data. All scores of a 0 (not present) and 1 (small amount present) were taken together as one group represented as a 0. All scores of 2 (moderate amount present) and 3 (large amount present) were also taken together as one group represented by a 1 (see table 2 in appendix V). Subsequently a logistic regression analysis was performed. Akaike's information criterion (AIC) was used for model reduction. AIC is used to select a model from a set of models. It compares the quality of a set of statistical models with each other by making a model for each variable of interest and rank them from best to worst. For the relevant effects (according to AIC) 95% (log-)likelihood profile confidence intervals were calculated.

According to the analysis only three effects were observed. In week 4 considerably more macrophages were present compared to week 1 and 2 (odds ratio 55, 95% confidence interval 6,1 – 4797,5). Also more collagen was present in week 4 compared to the other weeks (odds ratio 21, 95% confidence interval 2,8 – 482,9). In gel 3 considerably more inflammatory cells (in general) were

present compared to gel 1 and 2 (odds ratio 19,8, 95% confidence interval 1,9 – 873,3). The above-mentioned effects were independent of the type of sample. No relevant effects were observed between the microfracture group and the non microfracture group for the used parameters.

Discussion

The current study was performed to gain a better insight in the influence of microfracturing on fixation and degradation of a hydrogel in the early stages after surgery. It was suspected that that the microfracture technique stimulates healing of an cartilage defect by new blood supply and an inflammatory response, resulting in better filling with repair tissue and a better fixation of a hydrogel compared to a defect without a microfracture. But it was also suspected that microfracturing leads to an increased degradation of a hydrogel, as a result of the presence of phagocytic inflammatory cells, like neutrophils and macrophages.

The current study did not confirm a positive influence of microfracturing on fixation of a hydrogel and increased degradation of a hydrogel compared to no microfracturing. However, this conclusion has to be taken with considerably caution. This study was designed as an pilot study to find major differences within the results and to use the information of this study to direct future full-scale research projects. The scope of this project was to obtain information and to make decisions towards the two techniques. Unfortunately, it was not possible to answer the research question with the gathered data. So additional research is recommended to investigate the influence of microfracturing on fixation and degradation of a hydrogel, where this study could be used as a starting point.

The lack of sufficient data and the amount of test variables are important reasons for the failure to find significant differences between microfracturing and no microfracturing. 3 different hydrogels were used (hydrogel 1, 2 and 3) in this study in combination with 2 techniques (microfracturing or no microfracturing) and 3 different time points for euthanasia (week 1, week 2 and week 4). With a sample size of 9 horses, each horse has a different combination of test variables. For example, only one horse has gel 1 implanted and is euthanized after one week and only one horse has gel 1 implanted and is euthanized after two weeks and so on. This makes it difficult to ascribe a certain observation to the used technique, the used material or the used time point, because of a lack of different horses with the same set of variables. With a larger sample size or less test variables this problem could possibly be prevented.

Another possible explanation for the lack of clear differences is the used technique for the assessment of the samples. In this study the histological samples were evaluated for nine parameters, each of these parameters was scored on a scale from 0 – 3. The assessment of the samples and the subsequent scoring was performed by eye. So the results are based on the estimations and skills of the observer. For example, the study of Frisbie et. al. (2003), a comparable study, used digital analysis software for evaluation of the histological composition of the repair tissue and immunohistochemistry and PCR to detect collagen type II and aggrecan. Obviously these methods are much more reliable and sensitive to detect differences than assessment by eye.

As mentioned before no differences were observed between microfracturing and no microfracturing. It is already stated that this could be as a result of the study design, insufficient data, amount of test variables and assessment of the samples. However, also in literature significant differences are not

always found between the two techniques in the early repair stages. The abovementioned study of Frisbie et. al. (2003) is the only other study investigating the early response after microfracturing in the horse, with a special interest in collagen type II and aggrecan. In this study 12 horses underwent surgery to create full thickness cartilage defects in both stifles. In one of the two stifles microfractures were made, whereas the other stifle served as a nontreated control. Euthanasia of the horses took place at 2, 4, 6 and 8 weeks. Also in this study no significant differences were found between the type of repair tissue in the treated and control defects at week 2 and 4. At both time points the repair tissue of both groups contained a certain amount of collagen type II (detected with immunohistochemistry). The amount increased in the microfracture group after 6 weeks, but became only significant at 8 weeks. For the aggrecan content no significant difference was found between the 2 groups during the test period (Frisbie, 2003). A study with minipigs compared the repair of full thickness cartilage defects with and without microfracturing and full thickness cartilage defects with application of a bone marrow aspirate. Euthanasia took place at 4 weeks. No significant differences were found in the repair tissue of the three different groups, with the exception of a significant higher amount of subchondral bone in the full thickness defects without microfracture. Also immunohistochemistry for the detection of collagen type II did not show any differences, all defects contained a comparable amount of collagen type II (Gao, 2017).

Two other studies showed different results. In the study of Shortkroff et. al. (1996) 6 dogs underwent surgery to create chondral and osteochondral defects. Euthanasia took place at 2 and 4 weeks. The chondral defects showed no repair tissue at both 2 weeks and 4 weeks. The osteochondral defects showed variable results. One defect was completely filled, some defects were partially filled and some defects contained almost no repair tissue. With immunohistochemistry no collagen type II could be detected, only collagen type I (Shortkroff, 1996). In a comparable study with rabbits none of the defects without microfracturing showed any repair tissue at week 4 (the first time point for euthanasia). The defects with microfracture were filled with repair tissue containing chondrocytes, proteoglycans and collagen type II (Wang, 2011). A possible explanation for the different results is the removal of the calcified cartilage layer. The calcified cartilage layer was removed in the studies of Frisbie (2003) and Gao (2017), but it was not mentioned if that layer was removed in the studies of Shortkroff (1996) and Wang (2011). It is known that the best results for repair of chondral defects are obtained when the calcified cartilage layer is removed (Frisbie, 2006a). The possibly presence of the calcified cartilage layer in the last two studies could explain the absence of repair tissue in the defects without microfracturing.

The previously mentioned studies did only evaluate the influence of microfracturing on cartilage repair. They did not look at the influence of microfracturing on hydrogel fixation and degradation as in the current study. As far as known no studies have been performed to investigate the influence of microfracturing on fixation and degradation of a hydrogel in the first 4 weeks after surgery. In this study 3 different types of hydrogel have been used. Based on the results no clear differences have been observed between the three gels, with the exception of obviously more inflammatory cells in gel 3 compared to gel 1 and 2 (odds ratio 19,8, 95% confidence interval 1,9 – 873,3). This could possibly be explained by the composition of the gel. Maybe this specific composition makes is better detectable as a ‘foreign body’ than the other gels, resulting in attraction of more inflammatory cells. There are a bit more macrophages and neutrophils present in gel 3 (see table 1, appendix IV), as seen in a foreign body reaction, but not significantly more than in the other gels.

A gradual decrease over time in the amount of gel present in the defects was observed in all three gel types. In all the defects, initially containing gel 1 or gel 3, no gel was found at week 4, independently of microfracturing or not. The defects initially containing gel 2 did show a little amount of gel at week 4. This could possibly be explained by the inter-horse variations, because it is only one horse (horse 9) that shows this little presence of both gel and fibrin glue (control defects) at week 4. But this cannot be confirmed, because of the lack of other horses with gel 2 implanted and euthanized at week 4.

A striking observation in the current study was the presence of obvious bone erosion (score 2 and 3, see table 1, appendix IV) in the majority of defects (32 of 36), also in the defects without microfractures (15 of 18). In the defects treated with microfracturing it was suspected to find bone erosion as a result of the microfractures itself and the subsequent inflammatory response. The microfractures lead to disruption of the bone with subsequent necrosis. The following inflammatory response leads to attraction and activation of osteoclasts to digest and remove loose fragments and necrotic bone, resulting in bone degradation. However, this is a normal process in early fracture repair (Corrarino, 2015). In the defects without microfracturing it was not suspected to see extensive bone erosion, because the subchondral bone was left intact. The control defects without a microfracture contained only fibrin glue. Fibrin glue is widely used as an scaffold sealant and considered to be biocompatible and biodegradable (Jackson, 2001). Based on these properties it was not expected to see such extensive bone erosion as observed in the control defects. As far as known no studies in literature describe the same observation, so it is currently unknown what causes the bone erosion. The used fibrin glue in this study is of human origin, this may be the reason why it triggers an reaction. Maybe fibrin glue is not that biocompatible as expected in the horse. This could contribute to the observation of bone erosion in all the defects, since fibrin glue was used as an control and as an adhesive for the gel implants. Further research is recommended to investigate the possible influence of fibrin glue on bone degradation.

Another notable finding was the presence of eosinophils in 4 samples (see table 1 – appendix IV). However no clear pattern has been found in the presence of the eosinophils. They were observed in four different horses, at two different time points and in all three types of gel and one control. Eosinophils are produced in the bone marrow from multipotent hematopoietic stem cells. They are granulocytes with granules filled with cytotoxic proteins, lipid mediators, cytokines, chemokines and neuromodulators. Eosinophils are considered to be part of the innate immune system because of their ability for cytokine secretion and they play a role in the adaptive immunity by activating T-cells. Eosinophils are able to present microbial, viral and parasitic antigens as well as allergens to stimulate proliferation of T-cells. For this reason these cells are primarily known for their role in infections (especially against parasites and RNA virusses) and allergic reactions of the body (Blanchard, 2009; Rothenberg, 2006). However, they also react to tissue damage, inflammation, tumors and allografts (Rothenberg, 2006). Eosinophils can be recruited from the circulation to an area with inflammation or tissue damage where they secrete several proinflammatory cytokines. They are able to present antigens to trigger an immune response and they can release toxic proteins and lipid mediators from their granules to induce tissue dysfunction and damage. Beside this eosinophils are able to affect the properties of fibroblast by secretion of specific proteins or cytokines. For example, with secretion of TGF-β they stimulate fibroblast proliferation and collagen synthesis. Eosinophils also contain several matrix metalloproteinases and pro-angiogenic factors, so it is suggested that they possibly play a role

in modulation of extracellular matrix production and blood vessel formation (Muntiz, 2004). Their role in inflammation and tissue damage could explain why eosinophils were present in some of the samples. However, in that case it would be suspected to find eosinophils in more than just 4 samples. So further research is suggested to get a better understanding of their possible role in cartilage repair.

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Appendix I – Protocol for sample processing

Reagent	Station	Duration (minutes)	Temperature (°C)	Pressure/vacuum	Drain
Neutral buffered formalin	1	60	-	-	80
Ethanol 70%	2	59	45	-	80
Ethanol 80%	3	59	45	-	80
Ethanol 96%	4	59	45	-	80
Ethanol 100%	5	59	45	-	80
Ethanol 100%	6	59	45	-	80
Ethanol 100%	7	59	45	-	80
Xylene	8	50	45	-	80
Xylene	9	60	45	-	80
Xylene	10	60	45	-	80
Paraffin wax	Wax (I)	60	62	V	80
Paraffin wax	Wax (II)	60	62	V	80
Paraffin wax	Wax (III)	60	62	V	80

Appendix II – Protocol for hematoxylin & eosin staining

Chemicals and Materials

- Mayer's haematoxylin solution — Merck 1.09249.0500
- Distilled water
- Alcohol 96%
- Hydrochloric acid 37%
  - Distilled water — 400 ml
  - Alcohol 96% — 400 ml
  - Hydrochloric acid 37% — 4 ml
- Eosin — Merck 1.15935.0025
  - Eosin yellowish — 0,2 g
  - Alcohol 50% — 100 ml
  - Acetic acid — 1 drop
- Alcohol 50%
- Acetic acid
- Xylene
- Graded alcohol solutions (100%, 96%, 70%)
- Depex

Note: All these chemicals can be discarded in the cat. III chemical waste can (fluids) or bin (solids, e.g. filterpaper)

Procedure

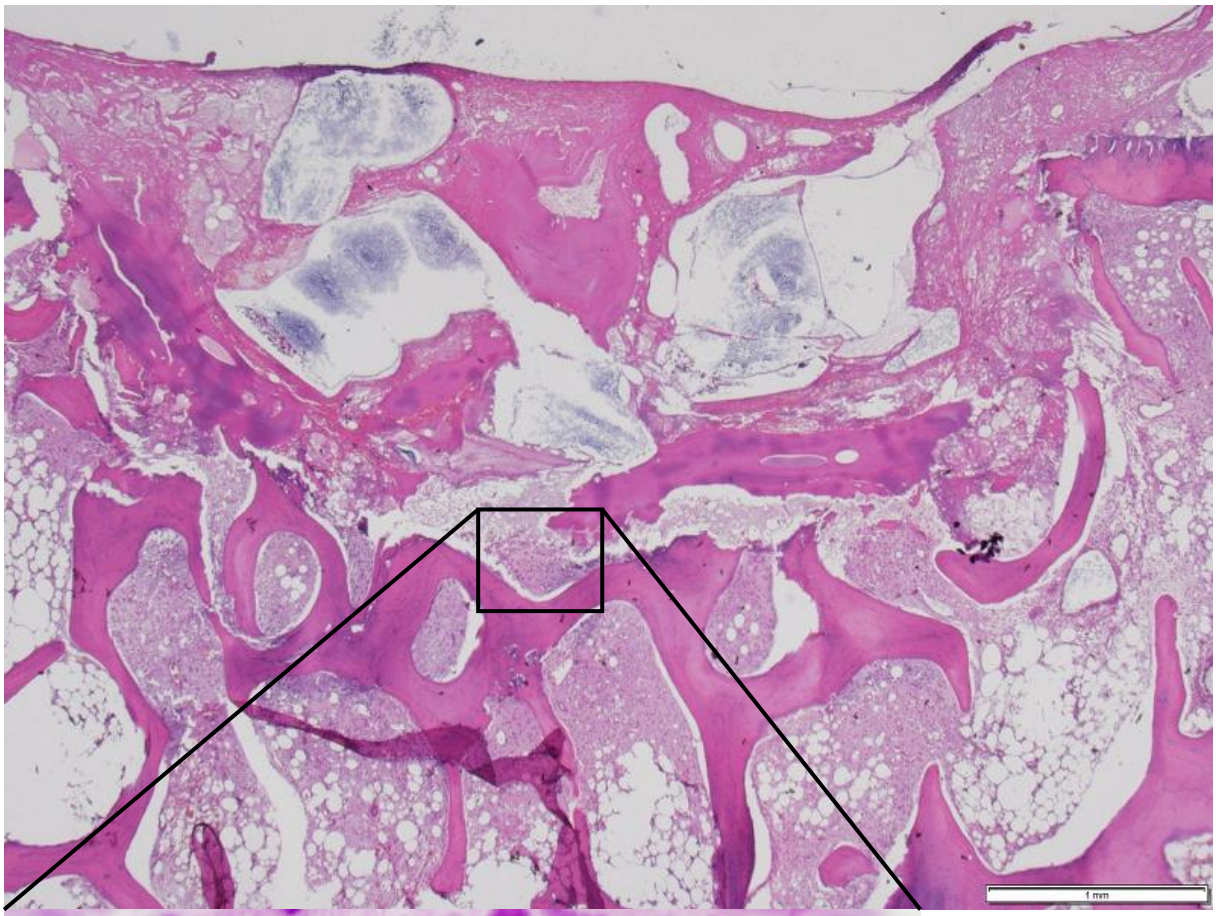
- Depraffinize sections according to SOP068
- Put your sections in distilled water — 5 min
- Stain sections in haematoxylin solution (filter before use!) — 10-20 sec
- Rinse the sections once with tap water and deposit this blue water as CA-III
- Wash well in running tap water — 10 min
- Check the staining. When haematoxylin staining is too blue de-stain your sections with hydrochloric acid-alcohol (dip) and wash again for 10 min. in running tap water. If not, you can skip this step.
- Stain the samples with eosin solution — 20 sec
- Dehydrate the samples with 70% alcohol 1x short in and out (eosin dissolves in alcohol 70%), 2x 96% alcohol short in and out, 2 x 5 min 100% alcohol, 2 x 5 min xylene
- Cover slip with permanent mounting medium (Depex)

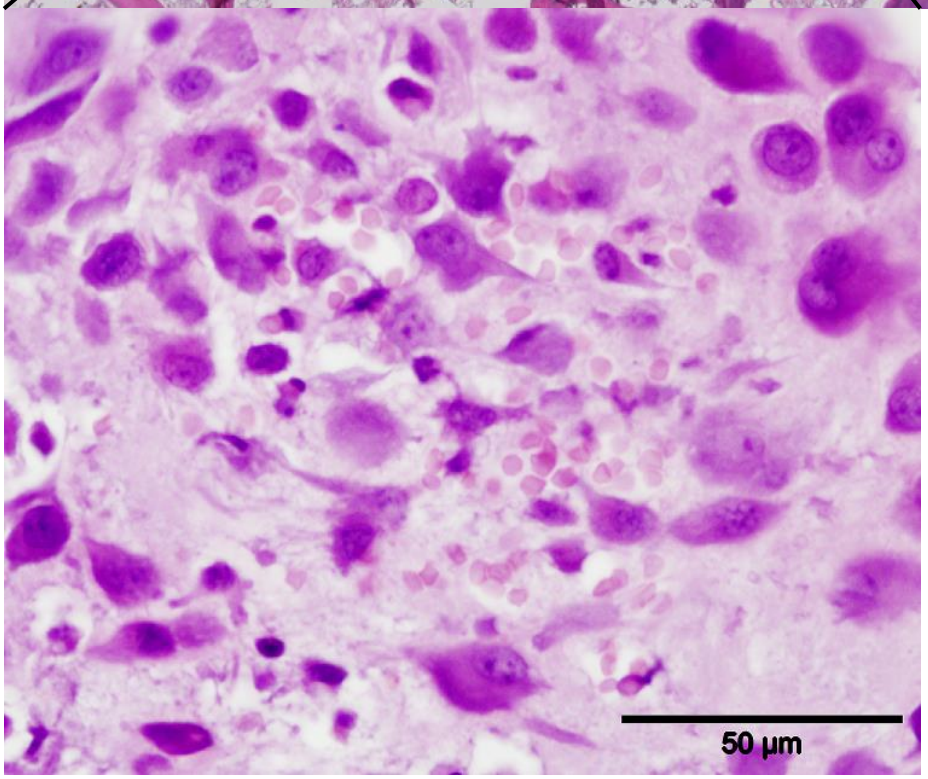
Appendix III – Examples of sample scoring

For the scoring of the histological samples a scale of 0 – 3 was used:

0 = no cells present
1 = few cells present
2 = several cells present
3 = many cells present

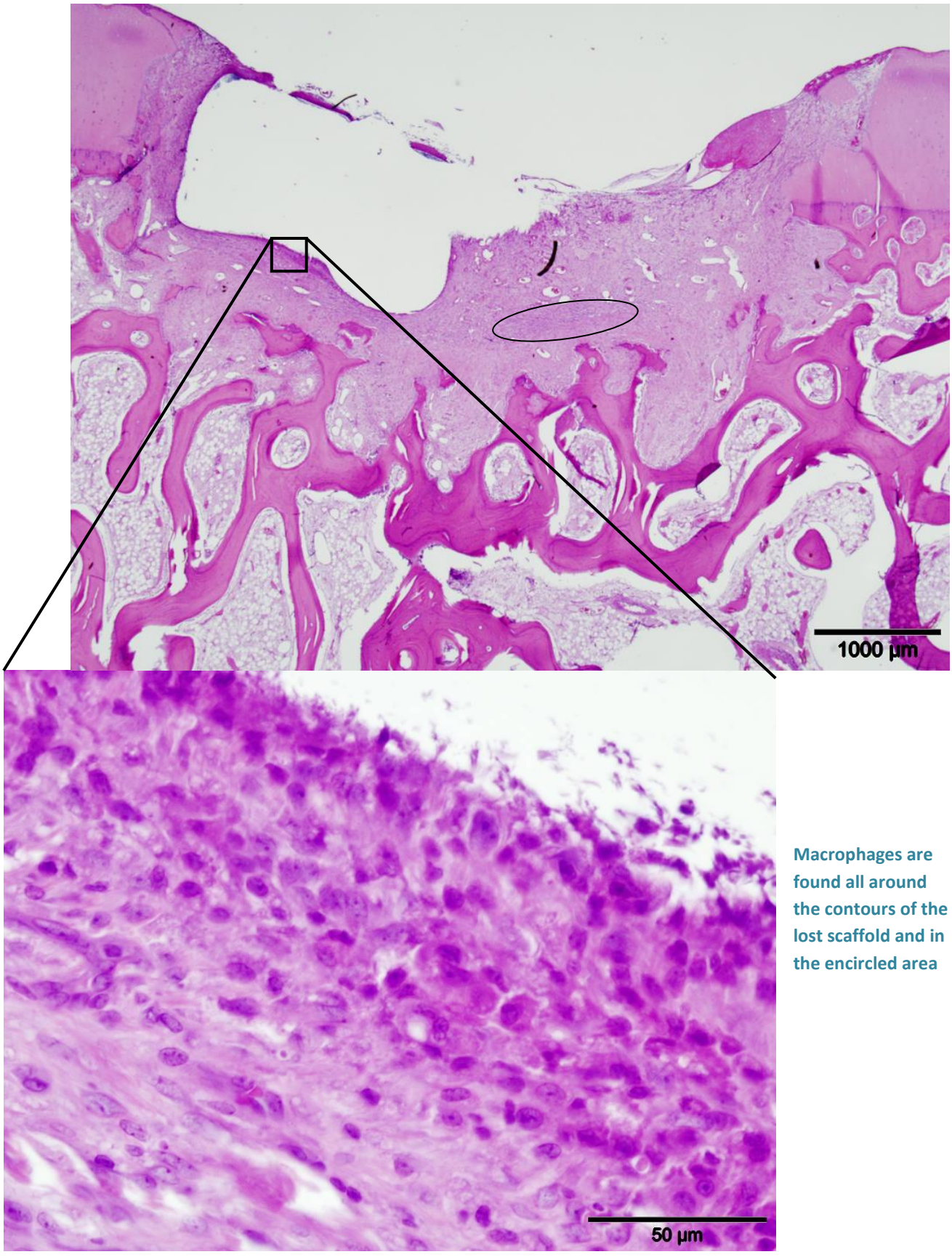
Score 1 – few cells present (gel 1 without microfracture, week 1)





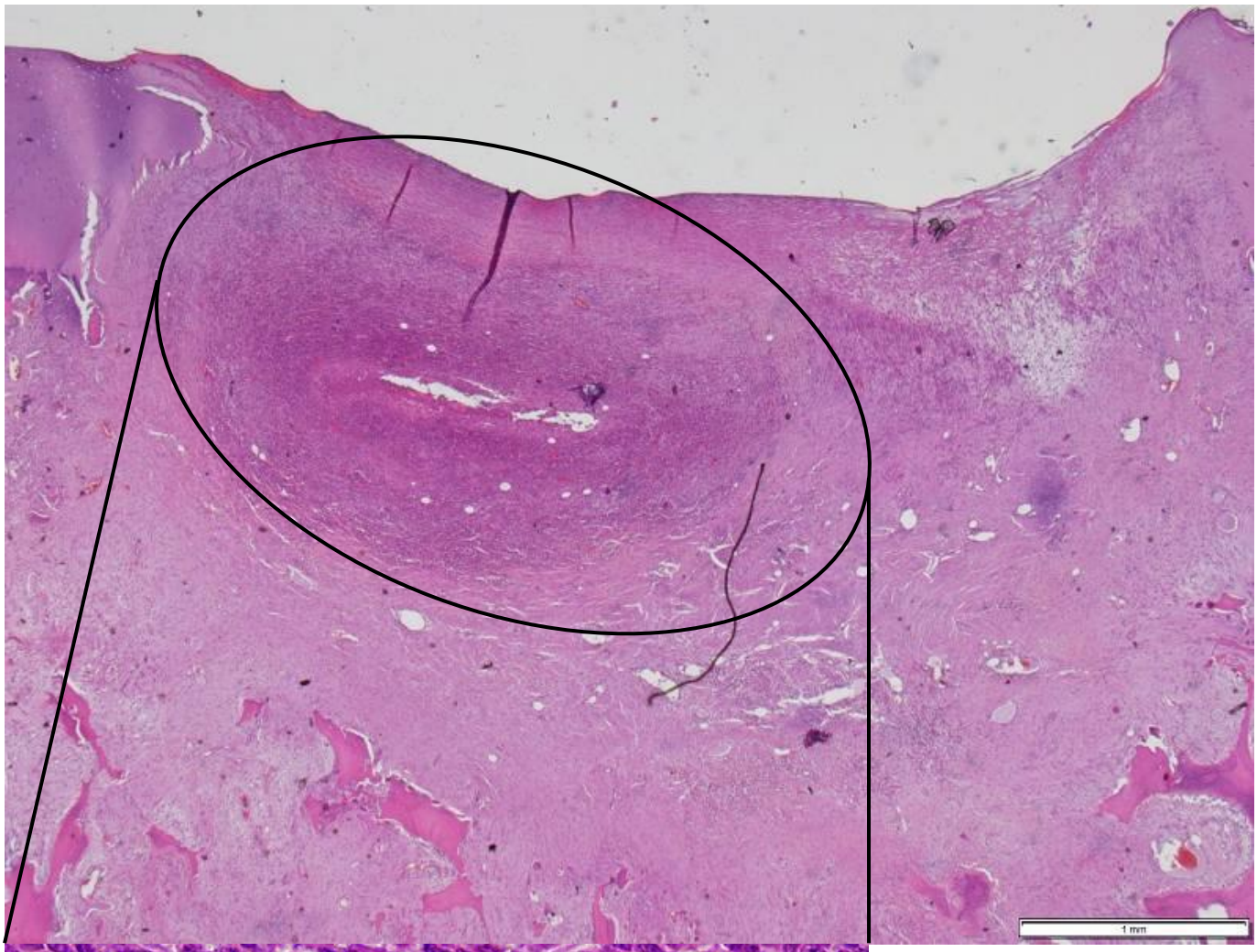
Only in this area of the defect some macrophages are found

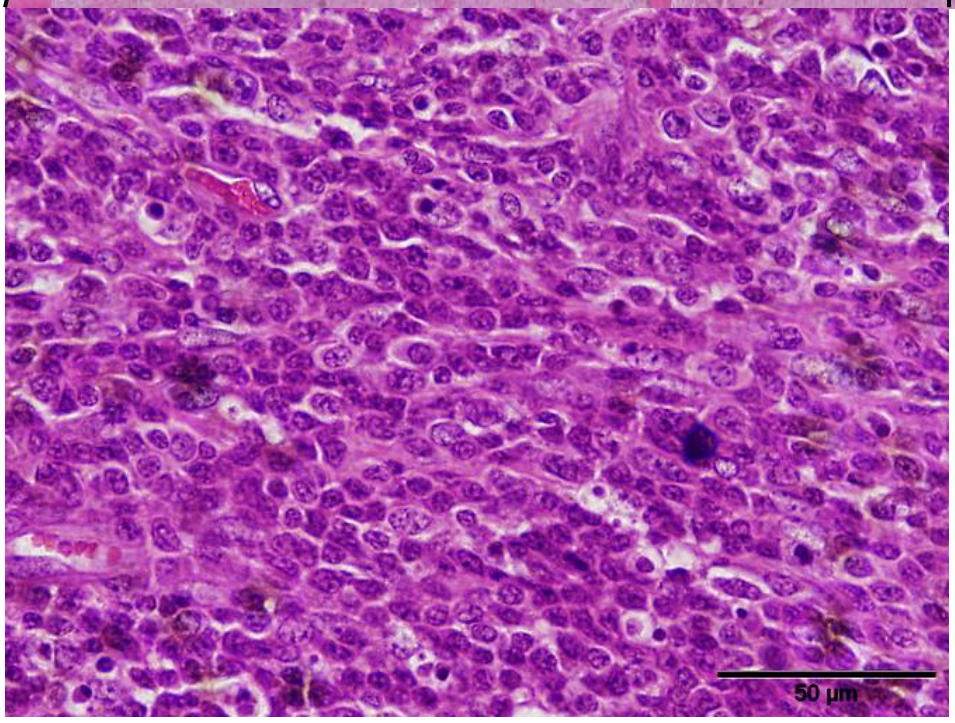
Score 2 – several cells present (Gel 1 without microfracture, week 2)



Macrophages are found all around the contours of the lost scaffold and in the encircled area

Score 3 – many cells present (control of gel 3 with microfracture, week 4)





In the whole encircled area a dense mass of macrophages are found

Appendix IV – Table 1

Horse	Sample	Reactive cells	Collagen	Capsule	Blood vessel	Amount of gel/fibrin glue	Bone erosion
6	***Gel 1 MFx – w1***	1 M: 1 - N: 1 - E: 0	1	0	1	2	3
4	***Gel 1 MFx – w2***	3 M: 2 - N: 3 - E: 0	1	2	1	2	3
2	***Gel 1 MFx – w4***	3 M: 3 - N: 0 - E: 0	2	0	0	0	3
6	***Gel 1 – w1***	1 M: 1 - N: 1 - E: 0	1	1	1	2	2
4	***Gel 1 – w2***	2 M: 2 - N: 0 - E: 0	1	0	2	0	2
2	***Gel 1 – w4***	3 M: 3 - N: 0 - E: 3	3	2	0	0	3
6	***Control 1 MFx – w1***	1 M: 1 - N: 0 - E: 0	2	0	0	2	2
4	***Control 1 MFx – w2***	3 M: 3 - N: 1 - E: 0	1	0	1	1	3
2	***Control 1 MFx – w4***	2 M: 2 - N: 0 - E: 0	3	1	2	0	2
6	***Control 1 – w1***	1 M: 1 - N: 1 - E: 0	0	1	0	2	2
4	***Control 1 – w2***	1 M: 1 - N: 0 - E: 0	2	0	3	0	3
2	***Control 1 – w4***	2 M: 2 - N: 0 - E: 0	1	1	0	0	1
5	***Gel 2 MFx – w1***	1 M: 1 - N: 1 - E: 0	1	1	1	2	2
3	***Gel 2 MFx – w2***	1 M: 1 - N: 1 - E: 0	0	0	1	2	3
9	***Gel 2 MFx – w4***	3 M: 3 - N: 1 - E: 0	1	0	0	1	3
5	***Gel 2 – w1***	1 M: 1 - N: 0 - E: 0	0	0	0	1	2
3	***Gel 2 – w2***	3 M: 3 - N: 2 - E: 3	1	0	1	2	3
9	***Gel 2 – w4***	0 M: 0 - N: 0 - E: 0	3	2	0	1	3
5	***Control 2 MFx – w1***	0 M: 0 - N: 0 - E: 0	1	1	0	1	1
3	***Control 2 MFx – w2***	1 M: 0 - N: 1 - E: 0	1	0	0	1	2
9	***Control 2 MFx – w4***	2 M: 2 - N: 0 - E: 0	3	2	2	0	3
5	***Control 2 – w1***	1 M: 1 - N: 1 - E: 0	0	0	0	3	1

3	***Control 2 – w2***	1 M: 1 - N: 1 - E: 0	2	2	0	2	2
9	***Control 2 – w4***	2 M: 2 - N: 0 - E: 1	1	2	0	1	3
7	***Gel 3 MFx – w1***	2 M: 1 - N: 2 - E: 0	0	0	1	3	2
1	***Gel 3 MFx – w2***	1 M: 0 - N: 1 - E: 0	2	3	0	1	3
8	***Gel 3 MFx – w4***	3 M: 3 - N: 1 - E: 0	1	0	2	0	3
7	***Gel 3 – w1***	2 M: 2 - N: 2 - E: 0	0	1	1	2	2
1	***Gel 3 – w2***	3 M: 3 - N: 1 - E: 0	1	1	1	1	3
8	***Gel 3 – w4***	2 M: 2 - N: 1 - E: 2	2	3	1	0	3
7	***Control 3 MFx – w1***	1 M: 1 - N: 0 - E: 0	0	1	1	2	3
1	***Control 3 MFx – w2***	0 M: 0 - N: 0 - E: 0	2	2	0	2	3
8	***Control 3 MFx – w4***	3 M: 3 - N: 3 - E: 0	2	3	1	0	3
7	***Control 3 – w1***	2 M: 2 - N: 0 - E: 0	0	0	1	2	1
1	***Control 3 – w2***	3 M: 3 - N: 3 - E: 0	1	2	2	1	3
8	***Control 3 – w4***	3 M: 3 - N: 0 - E: 0	2	2	1	0	3

Table 1 showing the result after scoring the samples for six different parameters (reactive cells, collagen, capsule formation, blood vessels, amount of gel/fibrin glue present and amount of bone erosion) at a scale from 0 to 3:

0 = not present
1 = small amount present
2 = moderate amount present
3 = large amount present
Gel 1 = Utrecht gel, Gel2 = Dresden gel, Gel 3 = Wurzberg gel
w1 = week 1, w2 = week 2, w4 = week 4
M = macrophages, N = neutrophils, E = eosinophils
Mfx = microfracture

Appendix V – Table 2

Horse	Sample	Reactive cells	Collagen	Capsule	Blood vessel	Amount of gel/fibrin glue	Bone erosion
6	***Gel 1 MFx – w1***	0 M: 0 - N: 0 - E: 0	0	0	0	1	1
4	***Gel 1 MFx – w2***	1 M: 1 - N: 1 - E: 0	0	1	0	1	1
2	***Gel 1 MFx – w4***	1 M: 1 - N: 0 - E: 0	1	0	0	0	1
6	***Gel 1 – w1***	0 M: 0 - N: 0 - E: 0	0	0	0	1	1
4	***Gel 1 – w2***	1 M: 1 - N: 0 - E: 0	0	0	1	0	1
2	***Gel 1 – w4***	1 M: 1 - N: 0 - E: 1	1	1	0	0	1
6	***Control 1 MFx – w1***	0 M: 0 - N: 0 - E: 0	1	0	0	1	1
4	***Control 1 MFx – w2***	1 M: 1 - N: 0 - E: 0	0	0	0	0	1
2	***Control 1 MFx – w4***	1 M: 1 - N: 0 - E: 0	1	0	1	0	1
6	***Control 1 – w1***	0 M: 0 - N: 0 - E: 0	0	0	0	1	1
4	***Control 1 – w2***	0 M: 0 - N: 0 - E: 0	1	0	1	0	1
2	***Control 1 – w4***	1 M: 1 - N: 0 - E: 0	0	0	0	0	0
5	***Gel 2 MFx – w1***	0 M: 0 - N: 0 - E: 0	0	0	0	1	1
3	***Gel 2 MFx – w2***	0 M: 0 - N: 0 - E: 0	0	0	0	1	1
9	***Gel 2 MFx – w4***	1 M: 1 - N: 0 - E: 0	0	0	0	0	1
5	***Gel 2 – w1***	0 M: 0 - N: 0 - E: 0	0	0	0	0	1
3	***Gel 2 – w2***	1 M: 1 - N: 1 - E: 1	0	0	0	1	1
9	***Gel 2 – w4***	0 M: 0 - N: 0 - E: 0	1	1	0	0	1
5	***Control 2 MFx – w1***	0 M: 0 - N: 0 - E: 0	0	0	0	0	0
3	***Control 2 MFx – w2***	0 M: 0 - N: 0 - E: 0	0	0	0	0	1
9	***Control 2 MFx – w4***	1 M: 1 - N: 0 - E: 0	1	1	1	0	1
5	***Control 2 – w1***	0 M: 0 - N: 0 - E: 0	0	0	0	1	0

3	***Control 2 – w2***	0 M: 0 - N: 0 - E: 0	1	1	0	1	1
9	***Control 2 – w4***	1 M: 1 - N: 0 - E: 0	0	1	0	0	1
7	***Gel 3 MFx – w1***	1 M: 0 - N: 1 - E: 0	0	0	0	1	1
1	***Gel 3 MFx – w2***	0 M: 0 - N: 0 - E: 0	1	1	0	0	1
8	***Gel 3 MFx – w4***	1 M: 1 - N: 0 - E: 0	0	0	1	0	1
7	***Gel 3 – w1***	1 M: 1 - N: 1 - E: 0	0	0	0	1	1
1	***Gel 3 – w2***	1 M: 1 - N: 0 - E: 0	0	0	0	0	1
8	***Gel 3 – w4***	1 M: 1 - N: 0 - E: 1	1	1	0	0	1
7	***Control 3 MFx – w1***	0 M: 0 - N: 0 - E: 0	0	0	0	1	1
1	***Control 3 MFx – w2***	0 M: 0 - N: 0 - E: 0	1	1	0	1	1
8	***Control 3 MFx – w4***	1 M: 1 - N: 1 - E: 0	1	1	0	0	1
7	***Control 3 – w1***	1 M: 1 - N: 0 - E: 0	0	0	0	1	0
1	***Control 3 – w2***	1 M: 1 - N: 1 - E: 0	0	1	1	0	1
8	***Control 3 – w4***	1 M: 1 - N: 0 - E: 0	1	1	0	0	1

Table 2 showing the results after converting the data into a binary scale
0 = not present or a small amount present
1 = moderate or large amount present
Gel 1 = Utrecht gel, Gel2 = Dresden gel, Gel 3 = Wurzberg gel
w1 = week 1, w2 = week 2, w4 = week 4
M = macrophages, N = neutrophils, E = eosinophils
Mfx = microfracture