Assessment of Biologic Resurfacing of Femoral Head in Dogs through Membranous Layer of Fetal Sheep Skull

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ABSTRACT

Objective: To present a new and effective method which can cause cartilage tissue recovery and to reduce the side effects of other surgical procedures. Methods: Twelve male and female dogs of at least one year of age were used in this study. The animals were randomly assigned to three groups of 4 animals per group. In group one, only the articular cartilage was removed. In groups two and three after removal of articular cartilage, the resurfacing was done using ovine fetal skull but in group three the hip joint was restricted using an ehmer sling. All animals were evaluated clinically for 60 days and macroscopic and microscopic evaluations were made after this time. Results: Clinical signs in groups one and two were better than animals in group three. In group three, lameness continued even after removing the ehmer sling until the end of 60 days period. In group one, the newly formed cartilage was incomplete in appearance. The articular surface in groups two and three appeared smooth macroscopically. In group two the cartilage was hyaline but without perichondral tissue but in group three scattered cartilage tissue with perichondral tissue was observed without any real resemblance to hyaline cartilage. Clinical Significance: Considering the result of this study, it seems reasonable to recommend the application of membranous ovine fetal skull to resurface the damaged articular cartilage of hip joint.

Key words: Resurfacing, Articular cartilage, Repair, Dog.

Introduction

The articular surfaces of bones are covered by hyaline cartilage, a specific form of supportive, connective tissue which is used to support and protect the bones. Since the hip joint bears the body weight and also animals make frequent use of this joint, the articular cartilage is destroyed and leads ultimately to severe lameness in some diseases. Besides its supportive functioning, the cartilage acts as a slippery and antipressure surface. When damaged, the healing process in this tissue is very slow, merely because of the lack of the vascular, lymphoid and nervous texture. Therefore, in the early stages of destruction, the animal feels no pain, but the permanent lesions will occur due to the continual use of the joint.

Many diseases related to joints such as osteoarthritis, osteochondrosis, the destruction of articular cartilage resulting from infection, trauma, tumors and rheumatologic disease are all surgical candidates, because medical treatment usually has no effects. In these cases routine methods such as reconstruction of joint, resurfacing arthroplasties and arthrodesis have been used for years.

Theoretically, it seems that the cartilage tissue is suitable for grafting. Fed by diffusion, this tissue has no vasculature and also has a relative proportion of
cell to matrix [7]. In recent years with significant progress in tissue engineering, different methods and techniques have been recommended to repair the articular surfaces: autografts, xenografts, periosteal autografts and cultured chondrocytes, which are all classified as biological resurfacing. Especially, the application of cultured chondrocytes as periosteal grafts has advantageous results [22].

In spite of our imperfect understanding of the treatment process of the articular cartilage, great efforts have been made concerning biological resurfacing methods. Methods which are used as biological resurfacing include: perichondrium transfer, periosteal transfer, osteochondral autograft and the recent autologous chondrocyte grafts [15]. Nowadays researchers work on engineering cartilage graft in order to inhibit the formation of fibrocartilage that results in pain and stiffness of joints [5]. In humans, cellular graft is clinically used to repair lesions in cartilage, but in animals this method is just experimental [13].

This study is an attempt to present a new and effective method which can cause cartilage tissue recovery. In this technique, a biologic graft was used to resurface the articular surface of the hip joint and consequently, to reduce the side effects of other surgical procedures. The theoretical underpinning of the method suggested in this article is that the membranous bone of the skull in sheep embryo has compatibility with the femur bone after grafting. Because of fetal xenograft, the possibility of graft rejection is low. After grafting to the femur head, the mesenchymal cells of the membranous bone of the skull are able to change to the articular cartilage with hyaline nature, and this hyaline cartilage can bear the mechanical stresses of weight better than fibrocartilaginous and also does not degenerate under stress. Furthermore, due to embryonic nature of this graft, the possibility of graft rejection is low and also in comparison with other biologic grafts such as autograft and allograft, this method is easy to manage, fast and financially economical.

Materials and Methods

The animals consisted of twelve adult male and female mixed breed dogs above one year old, weighing between 20 – 30 Kg. As a first step, the selected dogs received parasite medication routinely and the CBC was performed. After a complete clinical examination had been carried out, the dogs were numbered and randomly divided in three groups. Irrespective of their age and sex, each group consisted of four dogs. The operations performed in each group were as follows:

Group I: The articular cartilage of the femur head was erasured by orthopedic drills so that the bone under the cartilage could be seen. After the replacement of the femur head in acetabulum, the articular capsule was sutured even without resurfacing.

Group II: The articular cartilage of the femur head was erasured as above. Then it was resurfaced by the embryonic skull of sheep. With this, the femur head was replaced in acetabulum and then sutured. Both in Group I and in Group II, the joint was left unfixed so that it could have a normal, free movement.

Group III: After erasing the articular cartilage of the femur head and resurfacing by the embryonic skull of sheep, the joint was fixed in Ehmer fixation rout for 45 days. In this group, the joint fixation was performed in order to compare the effect of normal, free movement of the joint and the acceptance of xenograft.

Preparation of Skulls for Grafting

All the skulls were provided in an asepsis manner from the uterus of the slaughtered sheep in Slaughterhouse. The age of fetus was calculated by $X=2.1 (17 + Y)$, [X is the age of fetus in day; Y is the distance of head to sacrum (Rump – Crown)]. This was done just because of the possibility of the future use of the skulls if the age was suitable and if they were not calcified or were membranous. In the slaughterhouse, after separation of the head of fetus by scissors right in the junction of head to neck, the head was placed in a physiologic serum at 4°C in a small container which had an ice pack in order to keep the tissue cool and alive. It was quickly transferred to the operation room in a sterile fashion and was used in grafting within 6 hours. In the operation room, the skull was separated from the fetus head in the following manner:

At first, the skin of the skull was circumferentially cut with a scalpel. By the use of forceps and scissors it was completely removed from the skull and other tissues. Later the skull was cut circularly by scissors and separated from the brain and was used for grafting (figure 1). The age of fetuses ranged from 58 to 66 days and 4 fetuses were male and four were female. It is necessary to note that the shape of the skull bone, its diameter and convexity corresponded with the femur head. Being also compatible with the erasured articular surface, it was used for resurfacing the articular surface in grafting. The membrane around the skull was cut in order to create a circle graft corresponding with the femur head. It was not reliable to fix this graft without the fixation of the skull in the femur head. Therefore; in the second group in which the joint fixation was not carried out, the fixation of the graft was essential. For the fixation of the skull on the femur head a cerclage wire (no. 22) was used.
Preparation of Animals, Anesthesia and the Technique

All the dogs were prevented from having food and water for 12 hours before the surgery. After the administration of preanesthetic (Acepromazine 2%, 0.1 mg/Kg, IM), the operation site was prepared and disinfected routinely. The induction of anesthesia was performed by Thiopental sodium 2.5% (20 mg/Kg, IV) and was also maintained by Halothane 2% inhalation. The dogs were positioned at the right lateral recumbence so that they could be operated on their left joints. Before the operation, Cephazoline (40 mg/Kg, IV) was administered.

After the preparation of operation site routinely, a curved incision was made in the dorsolateral aspect of the hip joint on the skin. The fascia of biceps femuris muscle was cut and pulled to the caudal. The superficial gluteal muscle was cut from one fourth of the insertion to the lateral surface of the greater trochanter and then everted to the dorsal aspect. The proximal end of the greater trochanter was cut in the lateral aspect under the insertion of the gluteal muscle through osteotomy in the femur. Then, all of them were everted to the dorsal aspect. The blunt incision was also performed to indicate the caudal of acetabulum. Two protective sutures were placed in the articular capsule before the incision. The capsule was opened by the anchoring osteotom and haemostatic forceps. Then, the femur head was expelled from the acetabulum cavity. Finally, the cartilage of the femur head was destructed by the orthopedic drill (figures 2 and 3).

Fig. 1: Fetal sheep skull. Due to calcification, the middle skull cannot be used in grafting.

After removing and erasing the articular cartilage from the dogs in group II and III, two cross canals in the bases of the femur neck were made in order to fix the xenograft and to also prevent its dislocation. Two no. 22 cerclage wires with 15 cm length were passed through the canals. The wires are strained to form U shape (figure 4). Four ends of two cerclage wires are passed from opening produced in sides of skull. The two ends are tied together to form mattress horizontal interrupted suture or cress-cross suture (figure 5).

Fig. 2: Destruction of the femur head cartilage by the orthopedic drill.

Fig. 3: The orthopedic drill used in destruction of the femur head cartilage.

Fig. 4: Passage of cerclage wires from skull.

Fig. 5: Fixation of the skull on the femur head.
The resurfaced head of the bone was placed in the acetabulum cavity and the articular capsule was sutured with the absorbable synthetic vycril n.o.1. The osteotomized greater trochanter was fixed through the pin rout and figure of eight tension band wiring. The muscles were sutured by the absorbable synthetic vycril n.o in a continuous manner. The subcutaneous was sutured by the vycril no.0 and the skin was sutured by the monofilament nylon no.0 in a simple interrupted manner. During the operation, fluid therapy through the ringer lactated infusion was performed. At the end of the operation, the incision site was covered by the stent bandage. The Elizabeth necklace was provided for each animal to prevent them from licking the site and tearing the sutures. All the dogs were recovered from the anesthesia without any side effects.

**Post-operation Cares**

All the dogs were placed under intensive care postoperatively. Each of them was kept separately to prevent any local trauma. The postoperative cares included: daily inspection of surgical site; the injection of Ketoprofen 10% (2.2 mg/ kg, IM), the administration of penicillin G benzathin – procaine (2000 IU/Kg for 6 days) and also the administration of B complex (1 ml / 8 Kg weekly for 3 weeks). The compatibility and incompatibility of grafts after the fixation of the embryonic skull to the femur head were assessed and all the observations were recorded for two months.

**Study of Clinical Observation**

During the first week, the dogs were assessed with regard to the quality of the use of their feet, lameness, rotation, the quality of weight bearing and standing. In the second week, walking for a short distance (about 10 meters) was exactly tested.

**Assessment of Macroscopic Changes of Femur Head**

In day 60, the head of the femur was expelled from the acetabulum cavity. Then, the macroscopic changes of the cartilage, the quality of the xenograft, and the slipperiness of the articular surface of the femur head were examined.

**Assessment of Microscopic Changes**

After the examination, the head of femur was cut from the neck and the sampling was performed. The samples were placed in 10 % formalin and then in nitric acid in order for the bone to be decalcified and softened. Two different kinds of staining including h & E and verhalf were used to study the samples. All the dogs were euthanized by the administration of the intravenous Thiopental Na.

**Results**

**Results of Clinical observations**

In the first week, the dogs in all the three groups were examined daily and, then this continued on a weekly basis to ensure that no lameness was present. The dogs in group I did not show any weight bearing in the first three days, but in the fourth day, two dogs showed weight bearing on paw and the other two showed it on the tip of the paw. At the end of the first week, all the dogs in this group were able to bear weight on their legs and had only weak lameness. The complete weight bearing in this group started in the second week and towards the end of the week, they showed normal position and no lameness. No side effect, especially rotation to medial or lateral (toe out or toe in) was seen in this group.

In group II, the recovery process from clinical point of view and also the elimination of lameness were different. However, after two weeks from the operation, the very same recovery process was the case in this group as in group I. The large dogs began to bear their weight immediately after the operation on the tip of the paw, but in the small dogs no weight bearing was seen during the first week. In this group, the dogs were not allowed to walk. At the end of the second week, the large dogs were perfectly on their paw, but after walking for a short distance (about 10 meters) they showed lameness and used the tip of the paw for bearing weight. At the end of the day 21, lameness reached its minimum level. By the end of the fourth week in this group, lameness was completely eliminated. Forty days after the surgery, one of the dogs in the third group could run, but it showed mild lameness while running. However, this was eliminated fifty days after the surgery. No dog but one showed toe in one week postoperatively and no other side effect was reported.

In group III, the clinical observation differed significantly from the other two groups. In this group, the feet of all the dogs were fixed in an ehmer sling for 45 days. In day 45 and after removing the fixators, the dogs did not use their feet. In fact, they did not tend to move their feet at all. This situation continued until the end of the second month. The fixated feet also showed muscular atrophy.

**Results of Macroscopic Observations of Femur Head**

In group I, the growth of the cartilage tissue could be seen 60 days postoperatively (figure 6). In group II and III, through the macroscopic survey after 2 months, it seemed that the grafted skull was grafted with the femur and had been replaced with the normal cartilage. As a result, the growth of the
cartilage was complete and significant, and besides, it had resurfaced the articular surface of the femur (figures 7 and 9). There was no significant difference between the femur of the dogs in group II and III macroscopically, but the femur head in group III was not as adjacent and slippery as that in group II (figure 8).

Fig. 6: Macroscopic appearance of the femur head; day 60, group I.

Fig. 7: Macroscopic appearance of the femur head; day 60, group II. Note the junction of narrow piece of the articular cartilage with the grafted cartilage.

Fig. 8: Macroscopic appearance of the femur head; day 60, group III. Because of partially destruction of the articular cartilage, a piece of articular cartilage has been retained.

Results of Histological and Pathological Assessment of Femur Head

In the histological study of the embryonic skull of sheep which was provided for the graft as shown by figure 10, the calcification of the roof of the skull intramembranously, the peripheral bone lamina were not formed. Only were the trabecules forming, and between them in each side, mesenchymal tissue was present. With the growth of lamina, the greater laminas called trabecules were formed and these trabecules when joined together formed the spongiform bone. Mesenchymal tissue changed to periosteum and formed compact bone lamellae around the spongiform bone. Consequently, there was no cartilage model in skull. In this study in which a part of the skull was used for the graft, the skull had to be the mesenchymal or calcified mesenchymal model. In the skull, there is no normal cartilage formation, however, in the graft, metaplasia occurred and the hyaline cartilage in the mesenchymal tissue was formed.

In group II, the cartilage tissue was formed in epiphysis exactly like the articular cartilage which covers outside the surface of the epiphysis. This cartilage is hyaline and without perichondrium (figure 14). This is proven by the lack of the elastic fibers after the verhalf staining (figure 15). In this cartilage, the young chondrocytes, isogonics cells, territorial matrix and interterritorial matrix are distinguishable. The chondrocytes are enclosed by the capsule (figure 16).

In group III, the results were different. Pieces of cartilage are visible in a scattered fashion in the mesenchymal tissue of the skull in which some areas contain perichondrium, whereas in the articular cartilage, there is no perichondrium. This cartilage does not have any similarity to the articular cartilage. This may be due to the joint fixation. No fibrocartilaginous tissue was in the samples.

Discussion

Although great advances have been achieved in the treatment of articular diseases, there is no complete and successful method to treat some articular lesions, especially degenerative lesions. The anatomic structure and position of synovial joint differ from fibrous or fibrocartilage joints and
Fig. 10: Calcification of the roof of the skull intramembranously (H & E stain; ×30).

Fig. 11: Articular cartilage; group I. The resting, proliferative, hypertrophic cartilage and calcified cartilage zones and subchondral bone is seen (H & E stain; ×60).

Fig. 12: In this figure, articular cartilage of femur head has been removed completely to the level of subchondral bone (×60).

Fig. 13: The articular cartilage has been partially destructed and the mass of the compensatory cartilage resulted from the retained cartilage cells; group I (H & E stain; ×30).

Fig. 14: Grafted cartilage without perichondrium; group II (H & E stain; ×30).

Fig. 15: Grafted cartilage; group II. There are no elastic fibers. (Verhoef stain; ×40).

Fig. 16: Grafted cartilage; group II. The young chondrocytes, isogonics cells, territorial matrix and interterritorial matrix are distinguishable (H & E stain; ×100).

Fig. 17: Mesenchymal cells of the membranous fetal skull (right) are visible beside the distinguished cartilage (left); group II (H & E stain; ×100).
because of continuous mechanical stresses to these joints and bearing weight, these joints are more susceptible to degeneration. So, these lesions especially cartilage lesions need specific management. Most of inflammatory degenerative and non-inflammatory lesions of cartilage are reversible and if they occur in younger ages, the healing process is usually more rapid. Although several grafts have been used to heal and repair cartilage in human and animals [7,10,24,30], but the function of embryonic skull and its biological compatibility in grafting were questionable. The skull bone in human and animals is spherical and its size and shape are adjustable to surface femur. In the other hand, the embryonic skull heroically contains pluripotent mesenchymal cells which can be differentiated from the hyaline of fibrocartilaginous cartilage. Thus, it can be used to resurface the head of femur in adult humans and animals. This kind of grafting with an embryonic characteristic can be used to reconstruct the degenerated cartilage, especially in arthroplasty and arthrodosis. Since we used the embryonic skull to graft the head of femur with the erased cartilage, we can compare the results of this study with the other relevant studies. The clinical records in these 3 groups were of great significance. The beginning of weight bearing and rapid recovery of all animals in group I and II imply that there is no articular lesions and also local interaction in the healing process. The lack of anatomic lesions means that the involvement of the articular and the destruction of the articular cartilage do not impede the movement of the joint. In group III, not all the dogs could use their feet and it seems that it was the result of the external fixation of the joint and the limitation in movement and the rotation of joint. Seemingly, the fixation of joint is not suitable and is a factor for local changes and reactions. In this case, although graft is compatible, due to inflammatory reaction, the joint should not be fixated. In this study, graft compatibility was also proven.

In this study, histological changes in comparison with other studies were significant. In one study on the healing of the tissue of the cartilage of the hock joint in rabbits, the type II collagen was seen [2]. In another study on the normal joint of horses, the formation of cartilage in cartilage deficit with an increase in the thickness of the cartilage has been reported [12,23,29]. In this study, the mass of the compensatory cartilage resulted from the cell mass, which indicates that this area has an exceptional activity. In group II, the graft showed good organization, considering the clinical signs and histological changes in day 60 and the level of hyaline formed in this area. This is a very good result and also is due to the maintenance of the rotation field and the movement of joint. Some authors claim that, to some extent, cartilage formation results form the matrix flow or cartilage replacement [4, 6]. And some contend that this cartilage and fibrous tissue correspond with the lesion size and local healing reaction [14].

In this study, the homogeneity of cartilage was similar to the initial cartilage and this refers to the germinal activity of the mesenchymal layer of the embryonic skull that showed good compatibility in two months. We, still, do not know how our work will respond with continuous weighting and mechanical stresses. So, a longer period of study, minimum 6 months, is needed. In one study, grafting of the cultured homologous chondrocyte and the embryonic chondrocyte as epiphyseal rout in chicken filled the defects with hyaline cartilage completely [11]. In another study on rabbits, chondrocytes...
plunging in collagen gel were used and the defect was filled with hyaline cartilage containing collagen made from chondrocyte [31].

The mature cartilage does not usually heal with the natural tissue after destruction [1, 17] and the low possibility of healing in cartilage tissue allows for the use of other tissue grafts such as other mature cartilage tissue or cartilage bone pieces or the use of artificial joints [28]. It is obvious that the free movement of joint and weight bearing are the most important factors. It is reported that in rabbits, continuous passive movement is essential for complete healing of joints [26, 27] and in the other hand, it is obvious that normal weight bearing in the articular surfaces are necessary for the maintenance of cartilage [26]. In group III, the graft was not satisfactory and because of reduced rotation level and weight bearing, the muscular atrophy of foot occurred after two weeks and radiographic changes indicated that local changes are the factors to lameness.

In another study, it was recognized that the fixation of joint after periostem grafting for 3 – 6 weeks was an inhibitory factor to the synthesis of cartilage and it resulted in intra – articular adhesiveness [25]. Also, mobilization of joint after a period of fixation can cause differentiation in hyaline cartilage but this differentiation is not seen in animals which are immediately allowed to move. These recorded results are in line with the findings of this study. In our study, four dogs in group III cannot show weight bearing by day 60 because of adhesiveness, secondary changes, elevation of foot and lameness, whereas in some other studies, joint fixation after grafting periostem for 7 days was a better factor to accelerate recovery than a 14 day fixation period [3]. This does not seem logical and even these results are in disagreement with other reports. In one report, in the grafting of perichondrium femurotibial joint in 30 rabbits, the growth of new cartilage in continuous passive movement was better than fixation [20]. The continuous passive movement in comparison with active movement can cause an increase in the lifetime of new cartilage [19, 21]. The use of continuous passive movement in veterinary is usually impractical. Reduced collagen level, an increase in the transfer of collagen and also increased immature collagen are seen after the joints fixation.

Some experts believe that biological resurfacing has some problems such as insufficient strength to mechanical stresses and they can heal only limited lesions. They believe that the healing of osteochondral lesions with a diameter above 30mm with this technique is impossible, so they use new material with the same property of normal articular cartilage [22]. Sometimes osteochondral grafts are not suitable for large lesions in articular cartilage. Since there is limitation to obtain autologous osteochondral grafts, the orthopedic surgeons concluded that in some clinical cases they need several autologous osteochondral grafts to resurface large cartilage lesions [18].

In one study accomplished by Takeshi and coworkers, they concluded that to obtain better results it is necessary that the osteochondral graft be as large as lesions [16]. With regard to the obtained, and due to the fact that the embryonic skull can be adjusted with the articular surface and the lesion size, there will be no limitation in grafting in terms of the lesion size. Although the graft of chondrocyte is a useful medical strategy that results in the formation of repaired tissue like hyaline, the healing process may last for a long period. The guidelines of reconstruction in human undergoing the graft of chondrocyte include limitation in weight bearing for 6 months and the patient can go back to daily normal activities after 9 months. In horses, there is no significant healing following chondrocyte grafting after 4 months [8]. With regard to these results and the results of this study, it can be concluded that cartilage reconstruction by the use of membranous bone of embryonic skull can cause complete recovery in patients after a short time and has no important problem as other biologic routes.

The techniques of repairing cartilage by chondrocytes or other cell culture need external and in vivo cell culturing. Reconstruction following cartilage repairing by the use of cell culture may last for a long period and result in the limitation of movement of patients. In contrast, the technique used in this study lasts shorter and the patient can recover faster, because it does not need time for cell culturing. This is one of advantages of this technique in comparison with cell cultured routes.

The clinical examinations on human patients following perichondral or periosteal grafts have disappointing results. In these techniques, complete regeneration of hyaline articular cartilage does not exist and reconstructed tissues do not have stability for a long time. Moreover, there are many practical problems such as the isolation of grafts [9].

The retrospective analyses in the mosaicplasty technique have good results, but it may be said that mosaicplasty has more potential hazards [9]. With this study, it seems that there is no side effect which can threaten the articular health. It can be concluded that the mesenchymal model of the embryonic skull of sheep can differentiate hyaline cartilage and since this hyaline cartilage is more consistent than fibrocartilage and no fibrocartilaginous tissue was seen in these cases, the best result was obtained in group II.

As Robinson reported in 1998, mesenchymal cells are preferable to other cells to graft articular cartilage grafts [24]. It seems that this biologic xenograft can be a suitable alternative for the reconstruction and arthroplasty of the hip joint and other biologic resurfacing methods for the head of femur. It also has no side effect reported in such
methods. Since the membranous bone of the embryonic skull contains pleuripotent mesenchymal cells which can differentiate hyaline or fibrocartilage, the use of this graft before calcification of skull is recommended to reconstruct cartilage lesions in which cartilage damage is so large. It is better to use other methods to fix the skull on the femur head to reduce any local reactions by wires. With regard to the results in group II, it is also recommended that the joint fixation be avoided so that the cartilage tissue can be formed and local reactions can be prevented. Because of the geometrical figure of the embryonic skull and also provision of the sheep embryonic skull as biologic graft, it is highly recommended to establish a graft bank. As the results in this study were obtained in two months, a longer period of investigation for a minimum period of 6 months is also recommended in order to shed more light on the findings in the relevant research literature.

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