Pathological healing assessment of using fetal sheep amnion in experimentally induced bone defect rats

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Key words: Bone healing, amnion membrane, Histopathological, rat.

http://dx.doi.org/10.12692/ijb/5.5.8-15 Article published on September 05, 2014

Abstract

Rebuilding and renovation of lost bone whether because of physiologic or pathologic factors was one of the surgeons’ motivations from the past. Many researchers are trying to find materials that can improve bone healing in bone defect. The purpose of this study was to evaluate of histopathologically effects of fetal sheep amnion membrane on experimental bone defect healing in rat models. The experiment was conducted on 20 male adult SD rats. Animals were divided into two groups of control and experiment. After general anesthesia, a hole in size of 2×3 mm in diameter and depth was made using a dental bit in the inner aspect of the between condyles of left femur. In control group defect was left untreated and in experimental group, fetal sheep amniotic membrane was used to fill the bone defect. Histopathological studies for evaluation of bone healing were carried out in rats, which were euthanized after 45 days of the experiment using hematoxylin-eosin (H&E) staining method. In control group, defect seemed to be filled with adipose tissue with sparse hematogenic cells, in spite of a poor osteogenic activity and some osteoblasts could already be seen. In experiment group, many osteoblasts groupings, and Neo formed vessels occupied the gap, young bone trabecula increased in number and bone neoformation was more compact, with bone trabecula more organized. Due to osteo inductive properties of amnion membrane, provides a more rapid regeneration of bone defects. The results indicate that the amnion membrane is able to stimulate bone formation in rats.

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Introduction

Bone fractures in animals and humans are a major clinical problem that can be result of injury, illness, infection, bone tumors, bone cysts and etc. The common treatment methods involve external fixation by fixing the fractured bone or internal fractured bone fixation. However, these methods are not always successful in bone marrow inflammation and small fractures and bone tumors. However, studies of bone fracture healing indicate that time is an effective factor in the recovery process. These results are obtained by X-ray and mechanical examination of the fractured bone that in both types of experiments, the effect of the time was confirmed in fractured bone healing. So that the more rapid healing process causes to consistency and strength of the bone and reduces probability of bone transplantation or delayed transplantation and also infection and failure of orthopedic surgery (Blokhuis et al., 2000).

For doing so, use of spongy pieces transplantation or alternative materials is considered as a major orthopedic surgery method. However, despite its important effect of rapid healing, bone spongy transplantation has complications such as bleeding due to vascular injury and numbness due to nerve damage, hernias, chronic pain, infections, fractures regional imbalance in the pelvic area and etc. For elimination of these complexities different artificial alternatives of bond transplantation are used. These substances possess osteo-inductivity properties or act as a osteo-conductive agent as a carrier of bone proteins (Wolfe and Cook, 1993).

Fetal amniotic membrane is the innermost layer that composed of five layers of epithelium, basement membrane, dense layer, fibroblast layer and sponge layer. Amniotic membrane does not have blood vessels or nerve and nutrition is done by its surrounding tissues through diffusion. Amniotic membrane is more than one simple membrane without vessels; since it is active metabolically and plays an important role in transferring of solutions, liquids and biologic compounds such as vaso-active peptides, growth agents and cytokines Epithelial cells isolated from amnion have properties of the stem cells and the base membrane extracellular materials constitute a suitable environment for transplantation of cells and vessels. One of the thickest base membranes body tissues is amniotic membranes that protect fetal tissues during pregnancy. The base membrane is covered with dense fibrous tissue that collagen of this layer that is secreted by mesenchymal cells. Interstitial collagen (collagen types I and III) is the major constituent of the supportive tissue of the amnion. In addition, amnion membrane has other properties including anti-inflammatory, anti-microbial, anti-fibrosis, anti-scarring, mechanical and low immune stimulating properties (Samandariet al., 2011).

The aim of this research was to indicate histopathological changes and healing of experimental defects induced in bone of rat animal model after using sheep amniotic membrane.

Materials and methods

study protocols

The experimental study was conducted on 20 adult male Wistar rats (Sprague-Dawley SD) weighing approximately 250-300g in 2012 in the Department of Surgery, Faculty of Veterinary Medicine, Islamic Azad University of Tabriz. The rats were provided by experimental animals’ center in Faculty of Veterinary Medicine, Islamic Azad University of Tabriz. The rats were transferred to surgery ward and no experiment was conducted for one week in order to prevent any stress and compatibility with environment. The study protocol was approved by the ethics committee of the experimental animals. The rats were randomly divided into 2 groups, each with 10 rats. The first group or the control group received no treatment after bone defect. In the second group or the group treated with amniotic membrane, the bone defect was filled by sheep fetal amniotic membrane. The used amniotic membrane belonged to a sheep fetus under three months prepared from Tabriz industrial slaughterhouse transferred as sterile to surgery ward of the Veterinary Faculty. Amniotic membrane and
chorioallantois were removed carefully and amniotic membrane was washed by significant amount of sterile saline normal serum and placed in glycerol solution as cold protector and transferred to 4 °C until the tissue protective substances were absorbed and then transferred into the freezer with -80 °C. Before utilization it was placed at room temperature for 10 minutes.

**Preoperative preparations and anestheisa**

Preoperative abstinence was done for 4 hours and drinking water was prevented for 2 hours. For anesthesia, Ketamine 50 mg per kg body weight and Xylazine 5 mg per kg of body weight was injected intra-peritoneally. After induction of general anesthesia and the animal embedded mode positioning, operation part (inner region of the left femoral condyles) was shaved, washed and disinfected with Betadine solution and alcohol, then operation part was identified.

**Surgery**

All rats were surgically created bone defect in the same manner as follows. The used method was lateral patellar. At first an incision was created in 2 cm parallel to the femur length in the inner layer of the femur in distal part on the patella. Pushing the fascia and subcutaneous connective tissue and opening the joint capsule, the femoral condyle and the patella was exposed. By using dentistry drill in area between two condyles a defect was created in diameter of 2 mm and depth of 3 mm with turning speed of 6000 turns in a minute. So that at first a hole in diameter of 1m was created between two condyles by one drill and then a big hole in 2 mm was created by other drill. Byensuring surgical procedures, the site was washed with saline (Figure 1).

Then the defected area was left empty in control group and it was filled byamniotic membrane in the treatment group. Joint capsule was sutured in a simple continuous way by synthetic absorbable suture of 4-0 polyglicoalt produced by Supa Factory and the skin was sutured with single silk suture 3-0 produced by Supa Factory and the area was disinfected by Betadine and after a full recovery, the animals were transferred to special cages and were provided with food and water.

Post operation care: In order to prevent probable infections 6000 penicillin G was given muscular for 5 days and Celecoxib (Celecoxib 100 mg) 6 mg/w orally for five days . Every day, the swelling, inflammation and discharge or probable infections in the area of open sutures were studied.

**Histopathological evaluation**

in order to assess histopathology 45 days after surgery, the rats were first anesthetized by ether inhalation and then injected with a high dose of thiopental sodium (20 mg/ kg) intraperitoneally. Then an incision was done on the skin and muscles. The femoral condyle was removed and placed in 10% buffered formalin solution. The specification of each rat was recorded within the profile. After stabilization of the samples for a week, the bone tissue calcification was carried out. To perform this procedure, a solution of acid nitric 10% was used. After the end of calcification, the tissue preparation was performed for histopathological sections and the prepared slides were stained with hematoxylin and eosin method and then using light microscopy and histopathology they were studied.

**Results**

In microscopic observations of the experimental defect area induced between two condyles of the femur distal part in the control group, expansion of the articular cartilage tissue from the defect apical edges into inner section was clear that in the deep area it was converted to blade bone and filled and blocked completely the defect. The link between bone marrow and extracellular space was completely cut off by reconstructive tissue. The spongy blade bone fusion adjacent with bone marrow in the sides and the depth of the defect with new formed bone increased healing tissues from dense blade bone type in the defect area.

In microscopic studies of the experimentally induced
regenerated defect place between two condyles of the femur distal part treated with amniotic membrane, articular cartilage tissue extending from the apical sides of the defect into the inner section and conversion into the blade bone in apical defect and fusion of large amounts of the blade bone adjacent to the bone marrow from the sides and depth of the defect with newly formed bone in the defect depth was more severe than the control group so that a relatively thick and dense bridge in the defect depth blocked it. This area looks like a horse saddle similar to their natural state. In the depth of the defect, healing of the metaphyses area growth from defect by hyaline cartilage was completely identified.

**Fig. 1.**

**Discussion and conclusions**

As the main component in different animals, bone structure protects soft tissues and organs of the chest and skull. The tissue has calcareous material and forms the main body skeleton. Meanwhile it is active metabolically and plays an important role in regulation of calcium, phosphorus of blood and it experiences disposition and deformation. Moreover, these bones create leverage system that forces skeletal muscle contraction several times and convert the muscles contraction force to body movement. Regeneration of lost bone, whether physiological or pathological was one of the motivations for surgery. They were constantly seeking substance or method as a reasonable alternative in terms of both morphology and eliminating bone defects. Due to increasing bone trauma and injury and common limitations of traditional methods of healing and bone remodeling, new methods must be examined in this regard. Despite the great progress that has been made, but bone healing in orthopedic surgery due to other factors such as bone tumors, trauma or orthopedic surgery is a challenging problem. The treatment methods commonly used for fractures include external fixation without moving the broken bone or inner fixation of the broken bone. Despite this, the method such as non-fusion and delayed fusion, fractures, bone marrow edema and bone tumors are not always succeed. On the other hand, in places such as the hands or feet that overload is applied, fracture when walking or running, use of only implants in the fractured part can lead to failure of the implant. For this reason, using spongy pieces transplantation or alternatives is one of the most important methods in orthopedic surgery (Martinez and Walker, 1999).

**Fig. 2.** Microscopic view of the status of empirically regenerated defect place between two condyles of a rat femur distal part in the control group. Expansion of the articular cartilage tissue from the defect apical edges into inner section was clear that in the deep area it was converted to blade bone (narrow arrows) and filled and blocked completely the defect. The link between bone marrow and extracellular space was completely cut off by reconstructive tissue. The spongy blade bone fusion adjacent with bone marrow in the sides and the depth of the defect with new formed bone increased healing tissues from dense blade bone type in the defect area (thick arrows) (H & Estained, magnification × 40).

According to the importance of this issue the researchers study different factors in healing of the bone. It can be referred to spongy auto-transplantation. The use of bone transplantation is very common in cases where part of the bone tissue is...
lost due to surgery, accident or disease such as cysts and tumors of bone. For healing of the bone, the cells producing bone require to matrix for growth of the bone and accumulation of minerals and proteins. In the natural healing process of bone fractures, blood clots and connective tissue fill the space caused by the fracture and provide a suitable scaffold and structure for bone growth. If too much bone is lost, the more bone healing may be delayed. Continuous bridging in the fracture site among the fracture fragments and stimulation of bone healing process in the bone structure space are the best results of autotransplantation that is removed from the patient hip and transferred into the mentioned site (Ebraheim et al., 2001). However, in spite of the important effect in rapid healing spongy transplantation has complexities such as bleeding resulted from vessels and nerves damages, spactract, hernias, chronic pain, infection, fracture and regional imbalance in the pelvic area (Lekovic et al., 2002). In order to overcome these limitations different types of artificially prepared bone graft materials can be used. These materials induce osteo-inductivity or act as osteo-conductive agent as a carrier to transport proteins (Schmitz and Hollinger, 2001). Since 1980 onwards utilization of hydroxyapatite and other alloplastic materials has been common in various surgical procedures, especially in the mouth, jaws and face (De Wijs et al., 1993). Otherwise the properties of bone tissue formation can be named.

For the first time in 1910, Davis used fetal membranes for covering the site of transplantation. Stern and Sabella in 1913 independently began to treat burns and wounds of the skin surface using the amnion membrane. Subsequently, fetal membranes widely were used in the treatment of burns as surgical dressings of the mouth, bladder, vagina, and in tympanoplasty, arthroplasty, amnaloaceal and prevention of adhesions in the pelvis and abdomen (Trelford and Trelford-Sauder, 1979).

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**Fig. 3.** Microscopic view of the status of empirically regenerated defect place between two condyles of a rat femur distal part in the control group with high magnification. Expansion of the articular cartilage tissue from the defect apical edges into inner section was clear that in the deep area it was converted to blade bone (narrow arrows) and filled and blocked completely the defect. The link between bone marrow and extracellular space was completely cut off by reconstructive tissue. The spongy blade bone fusion adjacent with bone marrow in the sides and the depth of the defect with new formed bone increased healing tissues from dense blade bone type in the defect area (thick arrows) (H & Estained, magnification ×100).

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**Fig. 4.** Microscopic view of the status of the experimentally induced regenerated defect place between two condyles of a rat femur distal part treated with amniotic membrane in the treatment group, articular cartilage tissue extending from the apical sides of the defect into the inner section and conversion into the blade bone in apical defect and fusion of large amounts of the blade bone adjacent to the bone marrow from the sides and depth of the defect with newly formed bone in the defect depth was more severe than the control group so that a relatively thick and dense bridge in the defect depth blocked it. This area looks like a horse saddle similar to their natural state (narrow arrows). In the depth of the defect, healing of the metaphyses area growth from defect by hyaline cartilage (thick arrows) was completely identified (H & Estained, magnification × 100).
Amniotic membrane is a kind of medical residue that can be used after collection. Amnion membrane, the innermost layer of the placenta that is only one layer of epithelial cells attached to a thick base membrane and stromal matrix without vessel. Due to ability to reduce scarring and inflammation, acceleration of wound healing and epithelialisation and also having antimicrobial properties amnion membrane has been re-considered. Also, due to certain biological properties, recently is used as substrates for stem cell culture (Samandari et al., 2011).

Clinical comparison between control and experimental animals indicates that all animals have the same physical activity. The scars resulting from surgery on the leg skin was relatively low and uniform. In the control group, the defect was void hole that due to the absence of the state of irritating substance or growth of bone and connective tissue conversion into bone conduction and the intensity of healing was not so severe. According to the healing pattern in control group significant amount of fibrin thrombus and red cells fill the defect and so the vessels and fibrin tissue penetrate inside the defect. New bone formation is associated with the initiation of new vessel formation and progress towards its center. At the end it was observed that the defect area was filled and replaced with bone graft. Given the provocative nature of the amniotic membrane in the resection of pathologies such as carcinoma of the cornea and conjunctiva. So, amnion membrane as a good alternative is used in many ocular surface diseases. Amniotic membrane transplantation in acute and chronic chemical eye burns has been used for reconstruction of the conjunctiva and cornea. Amniotic membrane transplantation in acute and chronic chemical eye burns has been used for reconstruction of the conjunctiva and cornea (Shimmura et al., 2001). Researchers at Keio University in Tokyo show that using this membrane as heart cell membrane has possibility of 33 percent beating up and injection of these cells in the heart after two weeks of anfractuous increases the heart output 34 to 39 percent. The researchers believe that since this membrane is barrier between mother body and embryo and it rejected and according to theories these stem cells in this membrane could convert into other organs. The research on this theory continues.

Today, with advances in the sciences, the amniotic membrane is used in tissue engineering. Due to the special properties of amniotic membrane it has been proposed as one of the important sources to produce scaffolds. Processed amniotic membrane is used in eye burns (Shimmura et al., 2001). This substance is produced in the country.

The past decade has witnessed a resurgence of amniotic membrane in ophthalmology. Amnion membrane was used widely in treatment of corneal disorders such as neurotrophic ulcers, microbial keratitis, keratopathy and in cases of chemical burns. Also it was used for ocular surface reconstruction after
experiment group blade bones were organized orderly in comparison with the control group and the defect was filled with new bone cells. Previous research studies show anti-inflammatory effects of amniotic membrane (Solomon et al., 2005). Amniotic membrane involves epithelial cells leading to growth. It includes growth factors, anti-angiogenesis factors, angiogenesis, anti-inflammatory proteins, and natural protease inhibitors (Tseng, 2001). It also includes collagen type IV and VII, laminin 1 and 5, fibronectin and fibroblast growth factor (bFGF) (Rinastiti et al., 2006).

Previous studies show that the use of amniotic membrane can be effective in the healing of wounds, but there are very few studies on its effect on bone healing. Zhang et al (2004) suggest the positive effect of amniotic membrane in increase of bone (Zhang et al., 2004). Also, in other research Yen (2005) showed that stem cells extracted from amniotic membrane have been effective in nerve damage (Yen et al., 2005). In a study conducted by the use of amniotic membrane it was demonstrated significant effect in animal models of jaw injury. This study shows that according to the induction of fibronectin and laminin in the amniotic membrane it can be a suitable substrate for bone and jaw bone (Samandari et al., 2011).

In other study conducted by Yang and colleagues in 2010 the amniotic membrane was used in healing of tendon. This study showed the positive effect of this membrane in healing of tendon and formation of fibroblast and tendon fibers (Yanget et al., 2010). The studies refer to antimicrobial effect of amniotic epithelial cells in response to gram-negative bacteria and other inflammatory mediators in vitro excrete interleukin 6 and 8 and cause to rapid decline in the ability of the streptococci bacteria to survive in the laboratory conditions by adding in fetal membrane. It was observed lactoferrin and IL-6 inhibit the growth of Candida albicans(Epstein et al., 1998). In a research the feasibility of replacing human placenta with brain olfactory in a dog was studied and the results showed that amniotic membrane is a good alternative.

**Conclusion**

Study results indicate that sheep fetal amniotic can increase bone healing and bone properties and bone induction and it can be used in fracture.

**References**


