Histologic and histomorphometric evaluation of lyophilized amniotic membrane in bone healing: An experimental study in rabbit's femur

Nesma Mohamed Khalil
nesma_khalil27@yahoo.com

Lydia Nabil Fouad Melek

Contents lists available at ScienceDirect

Future Dental Journal

journal homepage: www.elsevier.com/locate/fdj
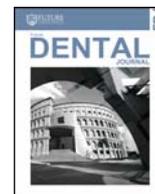
Histologic and histomorphometric evaluation of lyophilized amniotic membrane in bone healing: An experimental study in rabbit's femur

Nesma Mohamed Khalil[a,*], Lydia Nabil Fouad Melek[b]

[a] Department of Oral Biology, Faculty of Dentistry, Alexandria University, Egypt
[b] Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Alexandria University, Egypt

ARTICLE INFO

ABSTRACT

Amniotic membrane has been widely used in regenerative medicine especially ophthalmology. It has many advantages including anti-inflammatory, anti-fibrotic and antimicrobial properties. The purpose of the present work to study the effect of lyophilized human amniotic membrane on healing of bony defect in rabbits' femur. Eighteen male rabbits were used. Four mm wide and 5 mm deep defect was created in the femur diaphysis bilaterally in each rabbit. The defect of the right side was left empty (control), while the left side was filled with the amniotic membrane (study). Six rabbits were scarified at each of the experimental periods 2, 4 & 6 weeks postoperatively. The defect areas were dissected out and evaluated histologically & histomorphometrically. In the control group, at 2 weeks, woven bone spicules were seen extending from the periphery of the defect boundaries. At 4 weeks, the newly formed bone became more mature. At 6 weeks, the newly formed bone was more dense with newly formed osteons were seen. However, in the study group, the newly formed bone was much less in relation to the control group. Remnants of the amniotic membrane was seen folded at the center of the defect area surrounded by inflammatory cells. Histomorphometrically, the mean percentage of bone surface area in control group was higher than the study group in all experimental periods and the difference was statistically significant (p < 0.001). It was concluded that: freeze dried amniotic membrane is not suitable to enhance bone healing when used as a filling material in bone defects.

1. Introduction

Human amniotic membrane is the inner layer of the fetal membranes (the outer layer being formed by the chorion) and has been investigated as an alternative biomaterial in reconstructive surgery and wound healing. It was first used in 1910 by Davis [1].

Amniotic membrane use has expanded during the twentieth century especially as a dressing for burns and facial dermabrasions providing excellent coverage and healing potential in comparison to conventional materials [2,3].

Amniotic cells have been proved to produce several growth factors involved in wound healing as epidermal growth factor, vascular endothelial growth factor, and tissue inhibitors of metalloproteinase 1 and 2 [4].

That's why the biological and immunological properties of amniotic membrane have made it of special interest for use by clinicians in management of burn lesions, surgical wounds and ocular surface disorders [5,6].

Since the global awareness of virus transmission (e.g. AIDS) increased in 1980s, the use of human amniotic membrane decreased. However, by the end of the 1990s, new methods for the processing and cryopreservation of human amniotic membrane were established, and its use in wound care and reconstructive surgery became of interest once again [7]. In 2008, more than 2000 human amniotic membrane transplantations for ophthalmologic reconstructions were performed in Germany [8].

The amniotic membrane has demonstrated low immunogenicity as well as re-epithelialization, anti-inflammatory, anti-fibrotic, antimicrobial and antitumor properties. This is attributed to its ability to release biologically active substances, including cytokines and signaling molecules. These characteristics have recommended its wide use in regenerative medicine [9–12].

Interestingly, Wang et al. [13] have found that human amnion-derived mesenchymal stem cells stimulated increased levels of alkaline phosphatase activity (ALP), osteogenic marker genes, and matrix deposition, thus confirming that human amnion-derived mesenchymal

N.M. Khalil, L.N.F. Melek

stem cells can provide a preferential medium for driving osteogenic differentiation in bone marrow mesenchymal stem cells. These observations confirm the capability of human amnion-derived mesenchymal stem cells in the treatment of bone defects.

Most of researches which evaluated effect of amniotic membrane on bone healing used it as a covering for the bone defect [31] or in combination with bone graft [36]. So, the aim of the present work was to evaluate histologically and histomorphometrically the bone healing of the osseous defects (induced in the femur of rabbits) filled with the lyophilized human amniotic membrane in comparison to the negative control bone defects.

2. Materials and methods

The present study was approved by the Institutional Ethics Committee for Animal Use of Alexandria University. Eighteen New Zealand white male rabbits, about 5 months old, weigh 3–3.5 kg were used in this study. The left side was assigned as the study side and the right side assigned as the control side in each animal. Eighteen bone defects were filled with amniotic membrane folded in layers and the other eighteen contralateral defects were left untreated as negative controls.

Water and food were suspended for the animals 12 h before surgery. The animals were anesthetized with intraperitoneal injection of 30 mg/ kg ketamine. The anesthesia was maintained with Promazil (1 ml/kg).

The surgeries were carried out at the Institute of Medical Research, Alexandria University. First, shaving was done in the femoral diaphysis. An incision about 2 cm long was done on the distal surface of the femur using a scalpel blade No. 10. Then, a flap was elevated leaving the bone exposed. Drilling was done using surgical burs, to create bone defects in each femur of 4 mm diameter and 5 mm depth [31] (Fig. 1a&b). A sterilized ruler was used to check the dimensions of the defect, and a depth gauge was used to measure its depth.

The left-sided defect was filled with the freeze-dried sterile human amniotic membrane (Biomembrane; manufactured by National Center for Radiation Research, SAE) folded in layers (Fig. 1c). The right-sided defect was left empty as the negative control. The wound was sutured in layers, with absorbable suture of polyglactin. Afterwards, the rabbits were returned to their cages individually without mobilization of their extremities.

Analgesia was done with Diclofenac sodium (Voltaren75 mg/3 ml Solution for Injection) administered intramuscularly. Antibiotic (Cefotaxime: Cefotax 1 g, Egyptian int. Pharmaceutical industries co. Eipico) was administrated to rabbits for 5 days following surgery. Animals were monitored every day after surgery by the researcher, and veterinary technician.

Six animals were euthanized with an over dose of ketamine (KET A-100)at each of the experimental periods 2, 4 & 6 weeks postoperatively. The femurs of the right and left sides were obtained. The defect area were dissected out and processed for light microscopic examination and histomorphometric analysis.

2.1. Light microscopic examination

The defect areas were placed in 10% neutral buffered formalin for fixation, washed, decalcified in 10% EDTA, dehydrated in ascending concentrations of alcohol, cleared in xylene and embedded in paraffin wax. 5 μ thick sections were cut and stained with hematoxylin & eosin using conventional method [14].

2.2. Histomorphometric analysis

We have chosen to measure the percentage of newly formed bone because it is the most indicative parameter to assess bone healing [36] Morphometric evaluation of the newly formed bone in the defect area was calculated using Image J 1.46r program. Three longitudinal sections were cut from each specimen at different standardized depths. From each section, an image was captured using the same magnification power. In each image, three rectangles with standardized dimensions were drawn in three regions in the defect including the upper left border, lower left border and the center of the right border. The surface area of the rectangle was measured by choosing the region of interest (ROI) from tools and the measurement was recorded. Within each rectangle, the bone marrow spaces were selected using wand tracing tool, measured, and subtracted from the total area of the rectangle to obtain the area occupied only by bone (Fig. 2).

The results were expressed as percentage values (the proportion of area occupied only by bone in relation to the total area of the standard rectangle). The mean percentage of the newly formed bone of three rectangles in each section was calculated. The same procedure was repeated for each of the three sections of the same specimen and the mean was obtained. The same procedure was repeated for each of the six specimens in each group. The terminology used are those described by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [15].

Data obtained from histomorphometrical analysis were statistically described in terms of mean and standard deviation. Analysis of variance (ANOVA) test was used to compare the values between the different groups. Significance level (p value) was 0.05. Values less than 0.05 was considered statistically significant. Statistical analysis was completed using Statistical Package for the Social Sciences (SPSS) version 20.0 [16].

3. Results

3.1. Light microscopic results

3.1.1. After 2 weeks

Histological examination of the healing defect in specimens obtained from the control group revealed the formation of woven bone which consisted of intercommunicating bony spicules filling some parts of the defect. The bone spicules contained abundance of large osteocyte lacunae and were lined by osteoblast cells. Another part of the defect was filled with fibrous connective tissue. Cartilage like tissue was also seen in-between the newly formed bony trabeculae. Areas where active

Fig. 1. Application of amniotic membrane in the femur bone defect. a) The amniotic membrane, b) bone defect is created in the rabbit's femur, c) the defect is filled with the membrane.
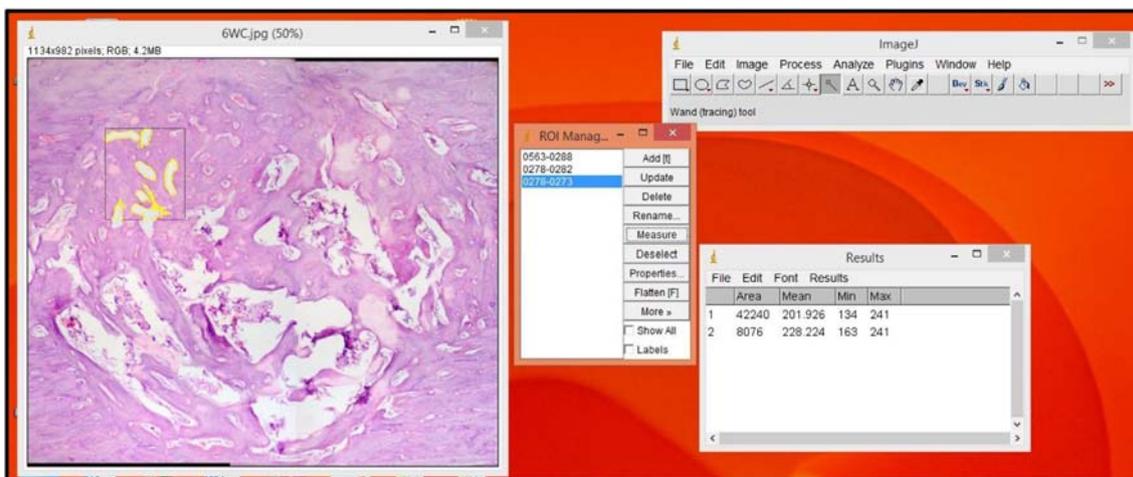
N.M. Khalil, L.N.F. Melek

Fig. 2. The surface area of the newly formed bone is measured using Image J 1.46r program. The bone marrow spaces are traced using wand tracing tool.
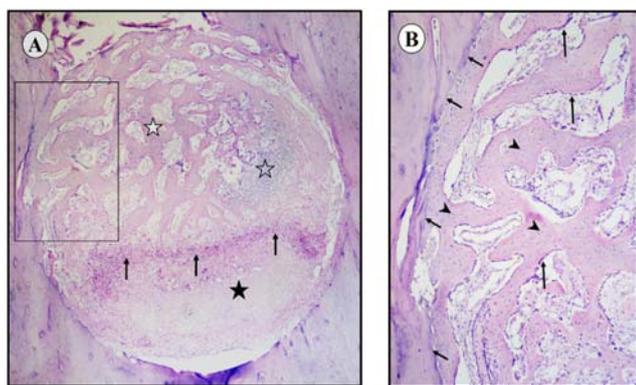

Fig. 3. Light micrographs (LM) of the defect site (2 weeks) in control group. **(A):** Compound LM showing some areas are filled with newly formed bone (white asterisk), while other areas is filled with fibrous connective tissue (black asterisk). Cartilage like tissue can also be seen (hollow asterisk). The area of active bone formation is represented with numerous osteoblasts (arrows). **B:** Higher magnification of the previous micrograph inset showing the newly formed woven trabeculae with rich osteocyte content (arrow heads) and a continuous layer of osteoblast cells (long arrows) lining the endosteal surface of bone. The borders of the defect site can be seen (short arrows). (H&E A: mag. x40, B: x100).

Fig. 4. LM of the defect site (2 weeks) in the study group. **(A):**Compound LM showing the defect site is filled with the folded amniotic membrane (white asterisk) and fibrous connective tissue (black asterisk). **B:** Higher magnification of the previous micrograph inset showing the amniotic membrane (long arrows) and the fibrous connective tissue (short arrow) containing some inflammatory cells. (H&E A: mag.40x, B: 100x).

bone formation was taking place were covered with large number of osteoblasts (Fig. 3A&B).

In the study group, the defect area was filled with folded amniotic membrane and fibrous connective tissue containing some inflammatory cells (Fig. 4A&B).

3.1.2. After 4 weeks

In the control group, the newly formed bone was seen filling a greater surface area of the defect in relation to the study group. The newly formed bone trabeculae consisted of mature lamellae containing regularly arranged osteocyte lacunae and lined by osteoblast cells. Complete osteointegration was seen between the newly formed bone and the old bone. At the central part of the trabeculae, darkly stained immature bone containing numerous osteocyte lacunae was recognized. At some parts of the defect the newly formed trabeculae were dense, while in other parts they were few and thin. The central region of the defect was empty containing scattered bone spicules and some inflammatory cells (Fig. 5 A&B).

In the study group, thinner and fewer trabeculae were seen growing from the defect boundaries. The adjacent part of the defect contained dense fibrous tissue and numerous inflammatory cells. At the center of
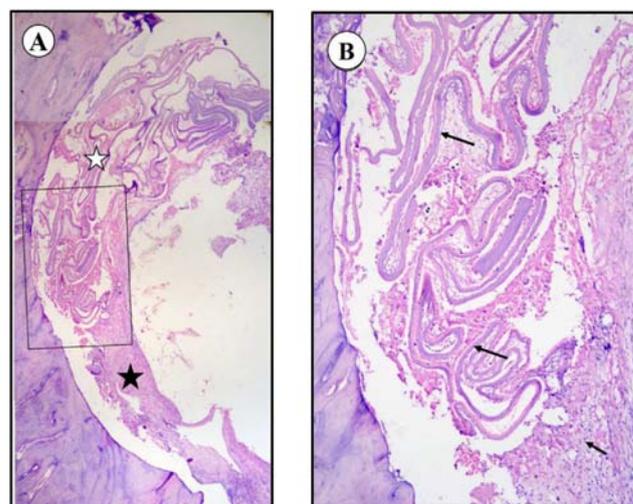
the defect, the remnants of the amniotic membrane appeared folded and surrounded by inflammatory cells (Fig. 6 A&B).

3.1.3. After 6 weeks

In the control group, the healing defect revealed the formation of more dense well vascularized bone occupying a larger surface area of the defect in comparison to the four weeks control group. Small osteons could also be seen. Areas of immature bone rich in osteocytes are present between mature bone trabeculae. Other areas of the defect showed formation of widely separated less organized bone trabeculae (Fig. 7A&B).

In the study group, the total amount of newly formed bone mass was much less in comparison to the control group. Thin bone trabeculae were seen extending from all around the defect boundary and lined by osteoblast cells. Remnant of the membrane still could be seen at the center of the defect in a folded pattern and surrounded by dense inflammatory cells (Fig. 8A and B).
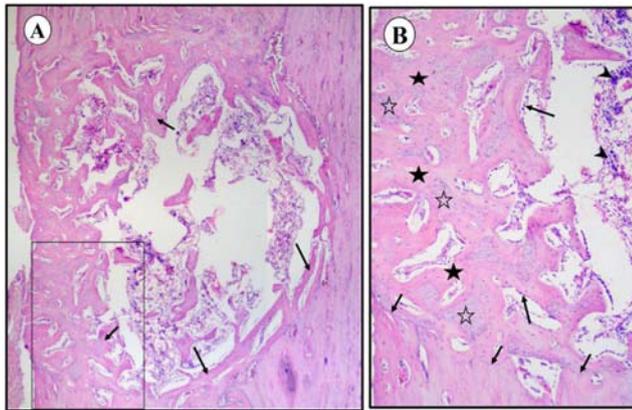
Fig. 5. LM of the defect site (4 weeks) in the control group. **(A):** Compound LM showing the bone trabeculae extending from the defect boundaries. In some areas the bone trabeculae are thin and few (long arrows), while in other areas the trabeculae are more dense and numerous (short arrows). **(B):** Higher magnification of the previous micrograph inset showing the organization of the bone trabeculae. The darkly stained woven bone (hollow asterisks) is surrounded by mature lightly stained trabecular bone (solid asterisks). Note the osteoblasts lining the outer surface of bone and endosteal surface of the bone trabeculae (long arrows), a line of integration between the newly formed bone & native bone (short arrows) and inflammatory cells at the center of defect (arrow heads).(H&E A: mag.40x, B: 100x).
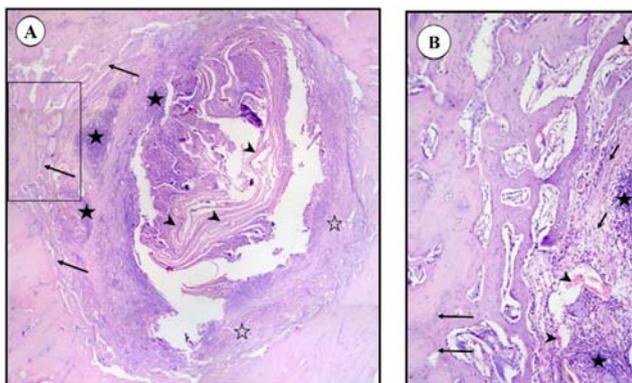

Fig. 6. LM of the defect site (4 weeks) in the study group. **(A):** Compound LM showing the newly formed bone trabeculae growing from the defect boundaries (arrows). The central region of the defect shows fibrous connective tissue arranged in a circular pattern (hollow asterisks) with some areas containing dense inflammatory cells (solid asterisks). Folded amniotic membrane (arrow heads) surrounded by inflammatory cells can be seen in central part of the defect. **(B):** Higher magnification of the previous micrograph inset showing the structure of the newly formed bone and osteointegration (long arrows) with the native bone. Note the adjacent fibrous connective (short arrows) containing dense inflammatory cells (asterisk) and newly formed blood vessels (arrow heads). (H &E A: mag.40x, B: 100x).

3.2. Histomorphometric analysis

The percentage of the newly formed bonein both study and control groups at 2, 4 & 6 weeks are summarized in Table (1) by means and standard deviation.

In control group, there was significant increase (p_1, p_2 & p_3 < 0.05) in mean percentage of bone surface area through all the 3 experimental periods where the values were 42.67 ± 3.56, 54.67 ± 5.39 & 69.33 ± 6.65 at 2, 4 & 6 weeks respectively. In addition, in the study group, there was also significant increase (p_1, p_2 & p_3 < 0.05) in mean percentage of bone surface area through all the 3 experimental periods where the values were 2.58 ± 1.07, 17.33 ± 4.72 & 30.33 ± 6.38 at 2, 4 & 6 weeks respectively.
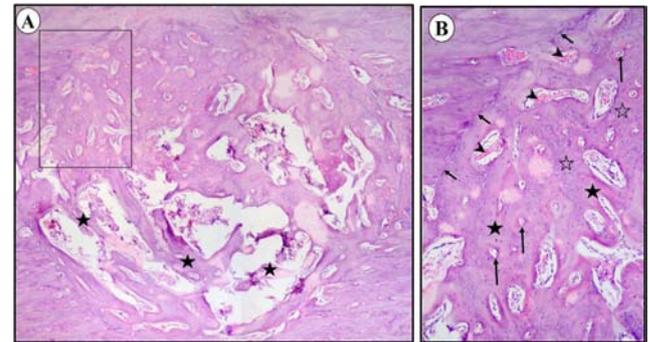

Fig. 7. LM of the defect site (6 weeks) in control group. **(A):** Compound LM showing the mass of the newly formed bone filing a large surface area of the defect site, while other areas contain widely separated trabeculae (asterisks). **(B):** Higher magnification of the previous micrograph inset showing the newly formed osteons (long arrows) and complete osteointegration (short arrows) with the native bone. The immature darkly stained bone (hollow asterisks) is surrounded by mature lightly stained mature bone (solid asterisks). Note the vascularity (arrow heads) of the newly formed bone. (H&E A: mag.40x, B: 100x).
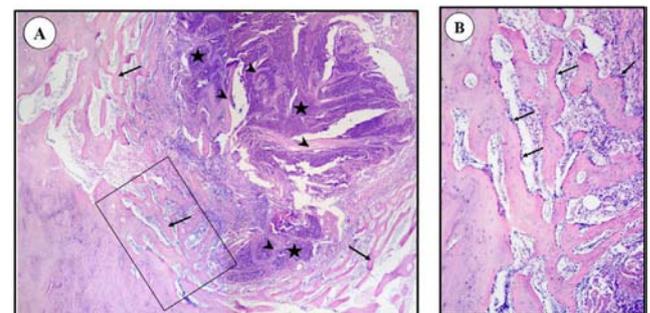

Fig. 8. LM of the defect site (6 weeks) in the study group. **(A):**The newly formed bone trabeculae are thin (arrows) and extending from the periphery of the defect towards its center. The central part of the defect contains remnants of the amniotic membrane (arrow heads) and dense inflammatory cells (asterisks). **(B):** Higher magnification of the previous micrograph inset showing the structure of the newly formed bone mass and plump-looking osteoblasts at the forming bone surfaces (arrows). (H&E A: mag.40x, B: 100x).

However, there was significant decrease in mean percentage of newly formed bone in study group in comparison to the control group in all experimental periods at 2, 4 & 6 weeks where p < 0,001.

4. Discussion

Amniotic membrane is derived from the internal layer of fetus membranes [11]. It has been used since 1940s as a natural scaffold material especially in ophthalmology [17]. It has many advantages including strength, translucency and flexibility. It contains many cytokines which gives the membrane unique anti-scarring properties [18]. Moreover, in clinical practice, amniotic membrane shows minimum inflammation and microbial infection [19,20]. Tamagawa et al., 2004 were the first to explore the pluripotency of cells isolated from human amniotic membrane [21]. They can differentiate into a wide variety of cells [22–24]. Therefore, use of amniotic membrane in regenerative medicine became a great promise.

Large bony defects are considered very serious complications that can result from accidents, invasive tumors and infections. If these defects didn't heal properly, serious health related problems may occur. So, choosing an appropriate graft material will be mandatory to stimulate new bone formation [25].

Kamadjaja et al. (2014) [26] demonstrated that mesenchymal cells isolated from human amniotic membrane express mesenchymal stem

Table 1

Comparison between the different studied periods and groups according to percentage of bone surface area (%).

Bone surface area (%)	2 Weeks (n = 6)	4 Weeks (n = 6)	6 Weeks (n = 6)	F	p
Control group					
Min. – Max.	39.0–48.0	47.0–60.0	60.0–77.0	34.351[*]	< 0.001[*]
Mean ± SD.	42.67 ± 3.56	54.67 ± 5.39	69.33 ± 6.65		
Median	42.0	56.50	69.50		
Sig. bet. periods.	$p_1 = 0.002^*, p_2 < 0.001^*, p_3 = 0.019^*$				
Study group					
Min. – Max.	1.50–4.50	11.0–22.0	20.0–37.0	53.239[*]	< 0.001[*]
Mean ± SD.	2.58 ± 1.07	17.33 ± 4.72	30.33 ± 6.38		
Median	2.25	19.0	31.0		
Sig. bet. periods.	$p_1 = 0.001^*, p_2 < 0.001^*, p_3 = 0.009^*$				
T	26.422[*]	12.764[*]	10.366[*]		
P	< 0.001[*]	< 0.001[*]	< 0.001[*]		

F, p: F, p values for Post Hoc test (**LSD**) for ANOVA with repeated measures for comparison between different periods.

t, p: t and p values for **Student t-test** for comparing between the two groups.

p_1: p value for comparing between 2 weeks and 4 weeks.

p_2: p value for comparing between 2 weeks and 6 weeks.

p_3: p value for comparing between 4 weeks and 6 weeks.

*: Statistically significant at $p \leq 0.05$.

cells surface markers (CD105 and CD90) and can differentiate into osteoblast cells when cultured in vitro in osteogenic medium. In addition, Go et al. (2016) [27] proved that amnion/chorion membrane extract can enhance the differentiation of cells cultured in osteogenic induction medium into osteoblast cells. This was due to upregulation of osteogenic gene expression (osteocalcin, osteopontin, runt domain-containing transcription factor and osterix), alkaline phosphatase activity and numerous growth factors including fibroblast growth factor and transforming growth factors. So the aim of the present work was to study effect of human amniotic membrane on healing of bone defect in rabbit's femur.

In our study, rabbits were used as an animal model. Rabbits are easy to obtain and house. Neyt et al. (1998) stated that rabbits are considered the first choice of animal models used in musculoskeletal research [28].Moreover, rabbits have a more rapid bone remodeling rate in comparison to primates and some rodents [29]. Wang et al. (1998) found that fracture toughness and bone mineral density of rabbits are very close to those of human [30]. In the present work, we have chosen the femur because its size is suitable to create bone defect and its accessibility for surgical procedure.

Histological results of the present study revealed that the defect site which were filled with the amniotic membrane showed much less bone formation in comparison to the control group. Few and thin bony trabecula were seen extending from the defect margins and the central part contained folded remnant of the membrane surrounded by inflammatory cells. Our findings contradict those of Ríos et al. (2014) who investigated the effect of Lyophilized amniotic membrane or collagen on bone defect healing in rabbit femur. They found that defects covered with amniotic membrane showed a higher bone density and new bone formation in comparison to those covered with collagen membrane [31].

In addition, Kerimog˜lu et al. (2009) found that human amniotic fluid can enhance tibia fracture healing in rats when instilled directly into the fracture line. They observed the highest score at the study group in comparison to the control. At the fifth week the fracture site was filled with woven bone and some cartilage. This can be attributed to the composition of the human amniotic fluid which contains hyaluronic acid many growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF),insulin-like growth factors I and II (IGF-I and IGFII) [32].

Amniotic membrane has anti-inflammatory properties.

Samandariet al (2011) found that human amniotic membrane can improve bone formation, decrease inflammation and exudate formation after vestibuloplasty in dogs [33].

Moreover, a clinical trial made by Kumar et al. (2015) to evaluate the use of amniotic membrane with bone graft in treatment of interdental defects. The amniotic membrane increased the amount of bone fill and decreased the inflammation indicated by decrease in the levels of interleukin 1β in gingival crevice fluid [34].

Interestingly, Go et al. (2017) examined the effect of each of amniotic and chorion membrane extracts (AME &CME) on osteoblast like cells differentiation in vitro. They found that both extracts can enhance the osteogenic differentiation of cells in vitro but the CME was more efficient. AME contained epidermal growth factor (EGF) which negatively regulated the osteogenic differentiation of cells in vitro. Co-culture of EGF withCME led to decrease in mineralization of extracellular matrix of osteogenic cells in vitro [35].

In our study the histomorphometrical analysissupported our histological results. There was significant decrease in mean bone surface area in the amniotic membrane group in relation to the control group. Contrary to our results, Starecki et al. (2014)found that the use of amniotic membrane can enhance new bone formation in femoral bone defect in rats. In defects filled with bone graft mixed with amniotic-derived tissue the amount of new bone formation was 49.2%, while in defects filled with bone graft alone, the newly formed bone was 37.8% only [36].

Our results contradict the previous studies which support the positive effect of amniotic membrane in enhancing bone formation. This may be attributed to several factors; firstly using the freeze dried form of the membrane in our study instead of the fresh one containing stem cells with regenerative potential. Also, the main and well known use of the amniotic membrane is in repair of wounds and burn lesions due to its re-epithelialization capacity rather than healing of bone defects. Moreover, the previous studies investigating its use in bone defects, have either used it in combination with bone graft [36]or as a covering membrane for the defect [31]. Using it as a sole filling material for the bone defect in the current study might have induced inflammatory processes leading to retardation of healing compared to the control group.

So, in conclusion our results don't support the initial hypothesis that the use of freeze dried amniotic membrane as a filling material may potentiate healing of bone defects. And we recommend further studies to either confirm or oppose our results.

Competing interests

The authors declare no competing interests relevant to this work.

Funding

No funding sources.

References

[1] Davis JS. Skin transplantation. Johns Hopkins Hospital Reports 1910;15:307–96.

[2] Walker AB, Cooney DR, Allen JE. Use of fresh amnion as a burn dressing. J Pediatr Surg 1977;12(3):391–5.

[3] Kucan JO, Robson MC, Parsons RW. Amniotic membranes as dressings following facial dermabrasion. Ann Plast Surg 1982;8(6):523–7.

[4] Park M, Kim S, Kim IS, Son D. Healing of a porcine burn wound dressed with human and bovine amniotic membranes. Wound Repair Regen 2008;16(4):520–8.

[5] Mermet I, Pottier N, Sainthillier JM, Malugani C, Cairey-Remonnay S, Maddens S, et al. Use of amniotic membrane transplantation in the treatment of venous leg ulcers. Wound Repair Regen 2007;15(4):459–64.

[6] Gomes JA, Romano A, Santos MS, Dua HS. Amniotic membrane use in ophthalmology. Curr Opin Ophthalmol 2005;16(4):233–40.

[7] Lee S-H, Tseng SC. Amniotic membrane transplantation for persistent epithelial defects with ulceration. Am J Ophthalmol 1997;123(3):303–12.

[8] Meller D, Pauklin M, Thomasen H, Westekemper H, Steuhl K-P. Amniotic membrane transplantation in the human eye. Dtsch. Ärzteblatt Int. 2011;108(14):243.

[9] Parolini O, Soncini M. Human placenta: a source of progenitor/stem cells? J. Reproduktionsmed. Endokrinol.-J. Reproductive Med. Endocrinol. 2006;3(2):117–26.

[10] Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, et al. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. Stem Cell 2008;26(2):300–11.

[11] Insausti CL, Blanquer M, Bleda P, Iniesta P, Majado MJ, Castellanos G, et al. The amniotic membrane as a source of stem cells. Histol Histopathol 2010;25(1):91–8.

[12] Koizumi N, Inatomi T, Sotozono C, Fullwood NJ, Quantock AJ, Kinoshita S. Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 2000;20(3):173–7.

[13] Wang Y, Jiang F, Liang Y, Shen M, Chen N. Human amnion-derived mesenchymal stem cells promote osteogenic differentiation in human bone marrow mesenchymal stem cells by influencing the ERK1/2 signaling pathway. Stem Cell Int 2016;2016.

[14] Drury R, Wallington E. Preparation and fixation of tissues. Carlet. Hist. Tech. 1980;5:41–54.

[15] Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units: report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 1987;2(6):595–610.

[16] Winer BJ, Brown DR, Michels KM. Statistical principles in experimental design. New York: McGraw-Hill; 1971.

[17] Sorsby A, Haythorne J, Reed H. Further experience with amniotic membrane grafts in caustic burns of the eye. Br J Ophthalmol 1947;31(7):409.

[18] Dua H, Rahman I, Miri A, Said D. Variations in amniotic membrane: relevance for clinical applications. BMJ Publishing Group Ltd; 2010.

[19] Hao Y, Ma DH-K, Hwang DG, Kim W-S, Zhang F. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. Cornea 2000;19(3):348–52.

[20] Sangwan VS, Basu S. Antimicrobial properties of amniotic membrane. BMJ Publishing Group Ltd; 2011.

[21] Tamagawa T, Ishiwata I, Saito S. Establishment and characterization of a pluripotent stem cell line derived from human amniotic membranes and initiation of germ layers in vitro. Hum Cell 2004;17(3):125–30.

[22] Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cell 2004;22(7):1338–45.

[23] Portmann-Lanz CB, Schoeberlein A, Huber A, Sager R, Malek A, Holzgreve W, et al. Placental mesenchymal stem cells as potential autologous graft for pre-and perinatal neuroregeneration. Am J Obstet Gynecol 2006;194(3):664–73.

[24] García-Castro IL, García-López G, Ávila-González D, Flores-Herrera H, Molina-Hernández A, Portillo W, et al. Markers of pluripotency in human amniotic epithelial cells and their differentiation to progenitor of cortical neurons. PLoS One 2015;10(12):e0146082.

[25] Li Y, Chen S-K, Li L, Qin L, Wang X-L, Lai Y-X. Bone defect animal models for testing efficacy of bone substitute biomaterials. J. Orthop. Translat. 2015;3(3):95–104.

[26] Kamadjaja DB, Rantam FA, Pramono C. The osteogenic capacity of human amniotic membrane mesenchymal stem cell (hAMSC) and potential for application in maxillofacial bone reconstruction in vitro study. J Biomed Sci Eng 2014;7(08):497.

[27] Go YY, Kim SE, Cho GJ, Chae S-W, Song J-J. Promotion of osteogenic differentiation by amnion/chorion membrane extracts. J Appl Biomater Funct Mater 2016;14(2).

[28] Neyt J, Buckwalter JA, Carroll N. Use of animal models in musculoskeletal research. Iowa Orthop J 1998;18:118.

[29] Castaneda S, Largo R, Calvo E, Rodriguez-Salvanes F, Marcos M, Diaz-Curiel M, et al. Bone mineral measurements of subchondral and trabecular bone in healthy and osteoporotic rabbits. Skeletal Radiol 2006;35(1):34–41.

[30] Wang X, Mabrey JD, Agrawal C. An interspecies comparison of bone fracture properties. Bio Med Mater Eng 1998;8(1):1–9.

[31] Ríos LK, Espinoza CV, Alarcón M, Huamaní JO. Bone density of defects treated with lyophilized amniotic membrane versus collagen membrane: a tomographic and histomorfogenic study in rabbit´s femur. J. Oral Res. 2014;3(3):143–9.

[32] Kerimoğlu S, Livaoğlu M, Sönmez B, Yuluğ E, Aynacı O, Topbas M, et al. Effects of human amniotic fluid on fracture healing in rat tibia. J Surg Res 2009;152(2):281–7.

[33] Samandari M, Adibi S, Khoshzaban A, Aghazadeh S, Dihimi P, Torbaghan SS, et al. Human amniotic membrane, best healing accelerator, and the choice of bone induction for vestibuloplasty technique (an animal study). Transpl. Res. Risk Manag. 2011;3:1–8.

[34] Kumar A, Chandra RV, Reddy AA, Reddy BH, Reddy C, Naveen A. Evaluation of clinical, antiinflammatory and antiinfective properties of amniotic membrane used for guided tissue regeneration: a randomized controlled trial. Dent Res J 2015;12(2):127.

[35] Go YY, Kim SE, Cho GJ, Chae S-W, Song J-J. Differential effects of amnion and chorion membrane extracts on osteoblast-like cells due to the different growth factor composition of the extracts. PLoS One 2017;12(8):e0182716.

[36] Starecki M, Schwartz JA, Grande DA. Evaluation of amniotic-derived membrane biomaterial as an adjunct for repair of critical sized bone defects. Adv. Orthop. Surg. 2014;2014.