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The Therapeutic Potential of Amniotic Membranes in Canine Femoral Iatrogenic Fracture Repair

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ABSTRACT: This study was to assess the therapeutic effects of amniotic membranes (AMs) in improving the healing of iatrogenic femoral bone defects in a canine experimental model. Ten male dogs were osteotomized to produce a standardized bone defect in the mid-shaft of the femur and randomly divided into a control group and an AM-treated group (n=5 dogs/group). Clinical and radiographic evaluation of bone healing was done on day 7 and 14 after surgery. The clinical observations showed a faster and full recovery in AM-treated group which had significantly less knuckling and evidence of discomfort than the control group. Radiographic observation on 7 days after surgery revealed that the bone defect was still evident with a dark radiolucency in the control group but in AM-treated group, there was already evidence of endosteal reaction around the defect. After 14 days, AM-treated group had shown vigorous callus differentiation resulting in a substantial reduction and almost complete disappearance of the defect, accompanied by a significant enhancement of callus density. Although the control group too exhibited the formation of the callus by the end of day 14 the defect was still visible slightly, which means that the healing path is slower. Together, they indicate that the use of the amniotic membrane is an effective method of accelerating and improving the quality of the bone repair in surgically-induced bone fractures of the femur and supports its promising ability as a treatment of canine orthopedic traumas.

INTRODUCTION

Long-bone fractures, and especially in the femur, are a threatening clinical complication in both human and veterinary orthopedic medicine, and commonly cause protracted recovery and disabling complications such as non-union, malunion, and chronic lameness (Żylińska *et al.*, 2021; Jassim *et al.*, 2023). Although contemporary surgical reduction and internal fixation have developed to a high level, biological bone repair is not optimal in the situations of large comminution or iatrogenic trauma (Hasenboehler *et al.*, 2011; Mavrogenis *et al.*, 2016). The case of iatrogenic fractures, namely fracture which occurs during surgical procedures such as screw or intramedullary nail drilling, is a special complication in which mechanical stress or thermal necrosis might weaken the integrity of bone (Chan *et al.*, 2025). Such fractures tend to happen in a surgically and biologically hostile environment, which requires new therapeutic approaches to improve and speed up the bone formation process to a higher level compared to the current state of the art practice. Herein the area of regenerative medicine provides a potential remedy in the form of amniotic membranes (AMs) which are ethically uncontroversial biological substances that

are obtained out of the inner-most layer of the placenta (Hu *et al.*, 2024). Such membranes have an enormous regenerative repertoire given their exclusive concentration of growth factors, cytokines and multipotent stem cells, which work together to produce strong anti-inflammatory, anti-fibrotic and immunomodulatory responses. Through the creation of conducive microenvironment, AMs can induce angiogenesis and differentiation of osteoprogenitor cells, and thus, directly affect the speed and quality of fracture repair. Although their therapeutic use is increasingly becoming popular, the precise effectiveness of amniotic membranes in treatment of iatrogenic bone fractures in a clinically relevant canine model has been critically under-investigated (Sanders *et al.*, 2024; Wells *et al.*, 2022; Tang *et al.*, 2018). Consequently, this study is designed to bridge this knowledge gap by evaluating the impact of amniotic membranes on the healing of iatrogenic femoral fractures in dogs, aiming to provide impactful insights into improving long-term clinical and functional outcomes.

MATRIALS AND METHODS

Animals

For this study, ten mature male canines weighing between 14 and 20 kg and between the ages of 1 and 3 years were chosen. For two weeks, the animals were kept in separate cages within the animal house and subjected to the same conditions, including feeding, management, and climate, in order to become acclimated to the new surroundings. To make sure they were healthy, these animals underwent radiographic and clinical examinations. Ivermectin 200µm/kg S/C was given to the animals for acclimation and administration were housed at the University of Basrah's Veterinary Animal House in Iraq.They were given regular dog food and water twice a day, in the morning and the evening.

Study design:

Dogs were split into two equal groups at random, with five animals in each group. Dogs were off fed and water about 12 h before operation. The operation was done after the animal aneasithezid by intramuscular injection of mixture from 5 mg/kg xylazine HCL (20 mg/ml from Alfasan in Woerden, The Netherlands) and 15mg/kg ketamine HCL (Ketamine Fresenius ®, Fresenius Kabi, Austria; 50 mg/ml) (Abduljaleel *et al.*, 2025). The control group were surgically operated were made under general anesthesia. After anesthesia, each dog's right hind limb was thoroughly clipped, shaved, and cleaned with soap and water prior to surgery. Ten minutes later, 70% ethyl alcohol and 2% povidin iodine were used to prepare the right hind leg aseptically. A 5-cm longitudinal skin incision was made on the limb's anterior-lateral side, and the muscle was laterally moved using forceps to reveal the bone. Ordinary osteotomies A special bone drill was used to create a 2 mm (limited size) bone hole in the middle of the femoral bone . The site of the drilling was irrigated with saline to prevent tissue from heating up the hole and these procedures were carried out in control. Following this, the muscle and skin were replaced, and the wounds were sutured with silk. 2% oxytetracycline wound spray was applied topically to the animals every day for five days after surgery.

After parturition, the pregnant cow's amniotic membrane was collected for the amniotic membrane group. The membrane rinsed three times in sterile physiological saline solution to loosen the clots, and occasionally gauze was used to remove the clots in between rinses. Decellularization and storage were place in PBS with 1% gentamycin at 4C. Following that, the same treatment was carried out as in the control group, but following osteotomies, a limited-size bone hole measuring 2 mm in diameter was created in the femoral bone's mid-shaft, prepare the amniotic membrane (sterile), to covered the hole of bone and fixed by 2-0 suture with muscle and then closed the skin with silk suture and complete the steps of operation .

The study was approved by the ethical committee of the University of Basrah's College of Veterinary Medicine in compliance with the BCVM standards. (Number 118/37/2024)

Post-operative care

For 21 days, the dogs were kept in stall rest and their clinical signs were checked every day. Penicillin and streptomycin, two broad spectrum antibiotics, were given intramuscularly for seven days at a dosage of 5 mg/kg B.W. additionally; OTC spray was used on the skin incision.

Radiological evaluation

At 7 and 14 days following surgery, radiographs of the fractures were taken under exposure fracture using 50 kv and 100 mA for 0.5 seconds. Assessment of callus development and the existence or absence of a hole were part of the radiographic

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evaluation. (With the AGFA CR 15-X x-ray equipment).

RESULTS

The canines showed no post-operative mortality. Through clinical and radiographic exams, the healing of femur hole fractures in the control and treated groups was assessed.

Post- operative clinical finding (at 7th and 14th days)

The control and treated group after recovery from anesthesia, in the early post-operative period, the dogs showed, pain, swelling in the site of operation and knuckling the right hand continuous for 21 days, and disappear the swelling at 7th days. It is worthwhile to note that the dogs in the amniotic membrane-treated group recovered more quickly than the control group; the swelling went away on the third day and the knuckling appeared on the fourteenth day. They also fed better and the surgical incisions healed on the seventh day after the procedure.

Radiological findings

The radiographic pictures of the dogs were taken seven and fourteen days after surgery. Seven days later, the hole is still apparent in the control group. Additionally, the hole's hue remains black and noticeable, and it does not go away (Fig. 1). The amniotic membrane-treated group has a higher density of endosteal callus development with a noticeable hole. Also, increase the callus formation of, periosteal and endosteal reaction with visible hole. (Fig. 2)

At 14 days, the control group's periosteal and endosteal calluses have formed, and the hole has somewhat disappeared. (Fig. 3).up the amniotic membrane-treated group, the hole vanishes, the callus fills up, and its density increases. (Fig. 4)

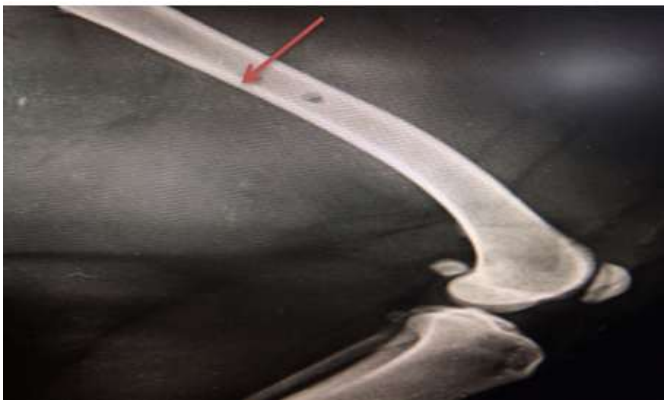

Figure- 1. Post-operative radiograph at (Day 7) show the control group (without amniotic membrane). The red arrow indicates to a visible defect/hole at the fracture site.

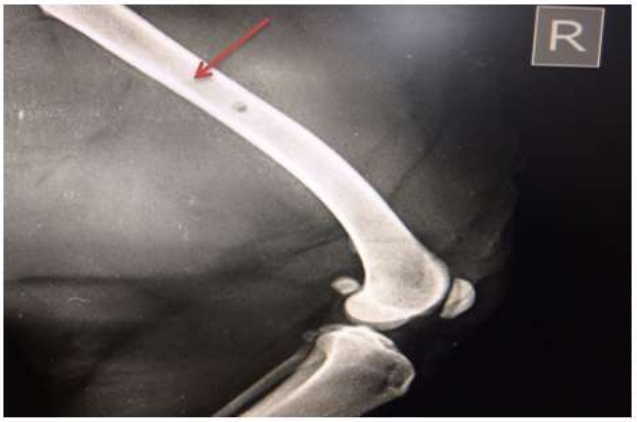

Figure- 2. Post-operative X-ray image shows at (Day 7) of the amniotic membrane-treated group. The red arrow points to the initial iatrogenic defect, now being integrated within the regenerative microenvironment.

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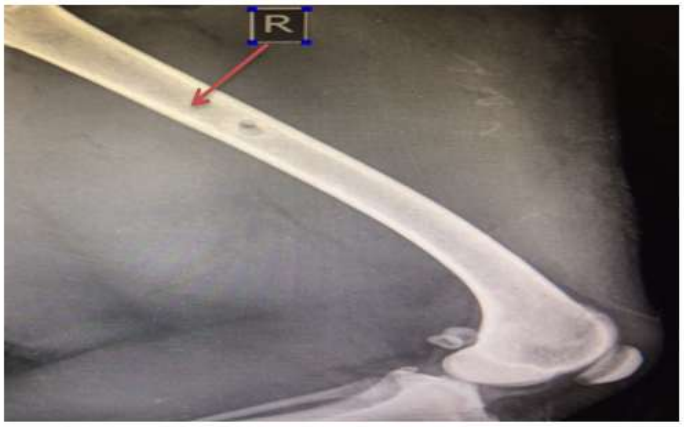

Figure- 3. Post-operative X-ray image at (Day 14) shows the control group (without amniotic membrane). The red arrow indicates to a slight reduction in the size of the cortical defect compared to day 7.

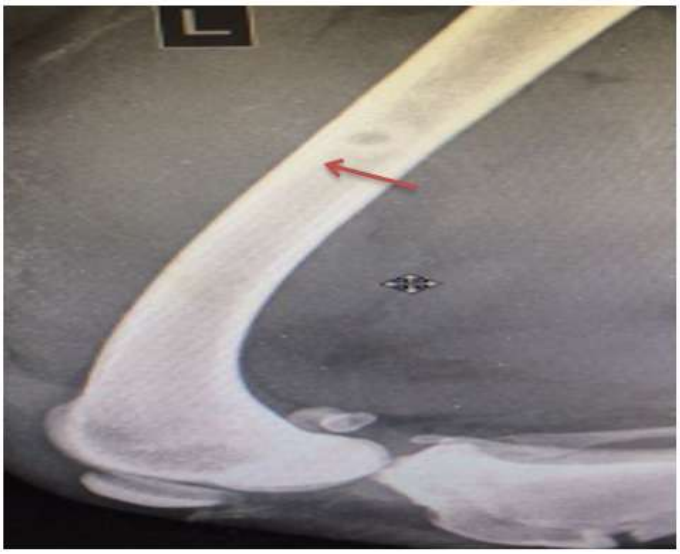

Figure- 4. Post-operative radiograph at day 14 shows the amniotic membrane-treated group. The red arrow highlights the surgical site, where the iatrogenic defect is being progressively filled with dense callus tissue, indicating advanced bone regeneration compared to control group

DISCUSSION

The present study investigated the therapeutic potential of amniotic membranes (AMs) as an adjunctive treatment for iatrogenic femoral fractures in a canine experimental model, evaluating their impact through clinical and radiological findings. The results of our observations have always shown a better healing pattern in groups that received amniotic membrane therapy than the control group, and this supports the immense benefits of using AM to quicken the healing process of fractures.

Clinical Manifestations of Enhanced Healing

The recovery profile of the dogs, which were treated with amniotic membranes, showed a significantly improved recovery profile during the experimental periods. Both treatment and control groups had the characteristic of pain, swelling, and knuckling at the place of surgery immediately after surgical intervention. These signs however were more resolved and faster resolved in the amniotic membrane-treated group. Particularly, localized swelling considerably decreased and completely disappeared by the 3rd day after the operation in the treatment group, which is the evidence of a rapid decrease of acute inflammatory response. Moreover, a functional impairment such as knuckling improved at a significantly faster rate, which

on the 14 th day, it had completely done in the treated animals. This clinical recovery was increased in better feeding patterns and faster wound healing in the surgical wounds and the wound was fully closed after the 7th day of the operation. These results also correspond to the previous studies that show the stimulatory effect of AMs on wound healing, which is probably explained by its ability to promote vascularization of the newly formed tissue, to activate the adjacent vessels, to enhance macrophage phagocytic capacity, and to induce the regenerating epithelium (Starecki et al., 2014; Hu et al., 2023; Jas The fact that the break strength of the bone increased by 14 days after surgery further proves the enhancement of structural strength of the healing bone in the treated group.

Radiological Evidence of Accelerated Osteogenesis

Radiological tests offered strong arguments to show the therapeutic prospect of amniotic membranes in enhancing osteogenesis. In the control group of dogs, the drilled bone defects (holes) were still clear and radiolucent (dark) and 7 days after the operation, which means that new bone formation was minimal. In sharp contrast, the amniotic membrane-treated groups experienced a significant growth in the place of periosteal and endosteal callus, even though the hole could be seen initially. By 14 days post-operative, a critical observation was the complete disappearance of the drilled hole in the treated group, indicating substantial infilling with callus and a discernible increase in callus density. While the control group also showed hole disappearance at 14 days, the quality and density of the callus formation in the treated group were superior. This acceleration of callus formation aligns with McClelland *et al* (2007) who noted that materials accelerating fracture healing are often identified by earlier radiographic evidence of bridging callus, even if the fracture line is not entirely obliterated. Our results strongly suggest that AM application effectively enhances the early stages of bone healing and mineralization.

Molecular Mechanisms Underlying Amniotic Membrane's Potential

The observed clinical and radiological improvements can be attributed to the well-documented biological properties of amniotic membranes. Bone formation, also known as osteogenesis, is a well-planned process that includes gene activation, osteogenic cell proliferation and differentiation, and the eventual mineralization of the extracellular matrix (ECM) (John, 2003) . The main cells that make bone, called osteoblasts, produce essential bone matrix proteins such type I collagen, osteocalcin, and alkaline phosphatase (ALP), which are subsequently calcified (Fetterolf and Snyder , 2012). The differentiation of osteoblasts is tightly regulated by various extracellular factors, including growth factors and cytokines, which modulate cell fate and play a vital role in bone regeneration and remodeling. While recombinant growth factors have shown promise, their clinical application faces challenges related to low yield, poor efficacy, and stability (Mohammadi *et al*., 2013; Yoon *et al*., 2017). Our findings support the concept that amniotic membrane acts as a robust biomaterial for stimulating cellular proliferation and differentiation, thereby directly promoting bone healing. Amniotic membrane-derived allografts contain a wide range of growth factors and cytokines which are naturally abundant and in large amounts relative to human embryonic stem cells or human bone marrow-derived mesenchymal stromal cells (Stacy et al., 2006; Kaka et al., 2016). This biologically avid environment enables the mesenchymal stem cell to be differentiated into osteogenic lineages, thus hastening the complicated bone repair cascade. In addition to the growth factors, AM is a biodegradable substance that has been known to promote healthy granulation tissue, anti-inflammatory and antibacterial properties and angiogenesis, which are all essential in facilitating the healing of fractures (Mohammed et al., 2015). The existence of the stem cells in the AM, which can asymmetrically divide and differentiate into osteoblasts, also adds to its regenerative ability when placed in a defect (Mohammed et al., 2015). Amniotic membrane, which is a derivation of extraembryonic tissue, has a unique structure consisting of epithelial layer, thick basement membrane and avascular mesenchyme (Sippel et al., 2001). Another enrichment of the regenerative potential of the membrane lies in the heterogeneous population of cells that the amniotic fluid alone harbors (Kai et al., 2018). This research is an amalgamation of amniotic-derived allograft material and bone graft, which can be viewed as an experimentation with a new type of technology that would allow improving orthopedic results. The natural presence of cytokines, growth factors, and hyaluronic acid in the amnion-based products makes them a very appealing alternative to the conventional autologous bone grafts, especially in the case of large bony defects (Marcus et al., 2008).

CONCLUSION

To summarize, radiographic analysis of the present study shows beyond any doubt that the healing of the bones in the group that was using amniotic membrane was much faster and qualitatively better than the healing in the control group. These findings are highly significant regarding the therapeutic value of amniotic membranes as accelerators and enhancers of healing

iatrogenic femoral fractures in a canine experimental model. This is a promising alternative that should be researched more to specifically identify its molecular mechanism of action that will lead to its possible inclusion in the orthopedic surgeons repertoire of improve healing of fractures.

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AUTHORS CONTRIBUTIONS:

All authors eqully contributed

CONFICT INTEREST*:*

The authors have declared no conflict of interest..

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