# Fresh Versus Cryopreserved Canine Amniotic Membrane: Physical and Histological Comparisons

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## 1. Abstract

The application of amniotic membrane (AM) in clinical practice is expanding all over the world. The most used forms of AM as regenerative scaffold are fresh and cryopreserved amniotic membranes. The present study was designed to compare the physical and histologicalfeatures of fresh and cryopreserved canine amniotic membranes. A total number of three adultfull-term pregnant female mongrel dogs aged approximately 3–5 years and weighing 20–25 kgwere subjected to elective cesarean section to get the amniotic membrane; then the animals AM divided into 2 groups: Group 1 (Amniotic membranes were preserved fresh in a mixture of saline and antibiotic in the refrigerator) and Group 2 (Amniotic membranes were preservedfrozen in -80˚c). The present results showed that fresh and frozen canine AM were similar in physical characters such as glistening, tensile strength, thickness, and transparency. The frozen membrane was turbid during thawing at room temperature and then restored its transparency after about 3 minutes. Histological findings of fresh amniotic membrane revealed the presence of all layer epithelium, basement membrane, compact layer, fibroblast layer, and spongy layer in an intactmanner resemble natural histological characters of amniotic membrane. On the other hand, preserved amniotic membrane showing damaged epithelial cells. From the present study, it could be concluded that the method of fresh amniotic membrane storage retained the morphological characteristics (basement membrane and stromal matrix) and epithelial cells viability which is usefully used as a potent source of stem cells and biological scaffold in regenerative therapy as in allograft. Meanwhile, cryopreserved amniotic membrane held its morphological characteristics, it lost the viability of the epithelial cells so it is recommended to use as a biological scaffold in cases with risk of rejection due to antigenic load of the tissueas in xenograft.

**Key words:** *Amniotic membrane (AM), Cryopreservation, Dog, Regenerative therapy, Stem cell.*

## 2. Introduction

The AM can be used "fresh," but in most countries, due to regulatory requirements. The AM must be kept for six months until a negative HIV screening result is confirmed. Several procedures have been devised to preserve AM for lengthy storage, including lyophilization and cryopreservation (by glycerol or Dimethyl sulfoxide (DMSO). The best preparation and preservation method is determined by the AM application and the required storage

time [1,2,3]. In soft tissue engineering, fresh AM (FAM) is commonly employed. [4,5,6].

FAM is a suitable amniotic product for tissue regeneration because of its native structure, intact growth factors, and live stem cells. Following isolation from FAM, both fibroblast and epithelial cells are viable, but these cells lose viability after isolation from cryopreserved AM (CAM) [7, 8].

The best technique to overcome FAM antigenicity is to decellularize AM. Decellularization, on the other hand, can reduce AM's thickness, mechanical properties, and immunogenicity while increasing its breakdown rate and safety [9, 10].

Decellularization agent urea is widely available. Through solubilizing proteins, urea detaches epithelial cells [11].

AM decellularization with urea is a simple and quick process.AM was treated for 5 minutes with 5M ice-cold urea, which fully removed epithelial cells while preserving ECM components and different growth factors. Another readily available decellularization agent is ethanol. Ethanol decellularization is a quick and safe method of decellularization. Ethanol isn't a particularly effective decellularized agent. After ethanol treatment, extra forceful scrapping can be used to accomplish successful decellularization. Decellularization of AM using 20% ethanol for 30 seconds followed by forceful scrapping indicated effective decellularization of AM with preserved ECM composition, basement membrane integrity, and growth factor expression. Due to the fact that ethanol cannot entirely dissolve epithelial cells, some epithelial remnants may remain following decellularization [12].

Air-drying AM is a very simple and quick way to preserve it. To make an air-dried AM (AAM), place the isolated AM on a stainless steel plate (epithelial side down, spongy side up) and place it in a sterile laminar flow (clean room) for 24 hours. Fill a double plastic bag with AM and seal it. If necessary, the packaged AAM can now be sterilized further [13,14,15].

In the European Union, cryopreservation of AM in 10% DMSO or 50% glycerol is a common approach for preservation of AM for tissue engineering and regenerative applications [16,17].

The cell viability in CAM samples was much lower than in FAM samples; however glycerol CAM demonstrated higher cell vitality than direct freezing. Longer storage duration improved tensile strength. CAM can be stored for 4 weeks and 6 months at - 20 ° C and -80 ° C, respectively [18,19].

The most commonly used forms of amniotic membrane as regenerative scaffold are fresh and cryopreserved amniotic membranes all over the world [7,20].

The present study was designed to compare the physical and histopathological features of fresh and cryopreserved canine amniotic membranes.

## 3. Materials and Methods

3.1. Animals:

A total number of three adult full-term pregnant female mongrel dogs aged approximately 3–5 years and weighing 20–25 kg were subjected to elective cesarean section to location to get the fetal amniotic membranes; then the animals AMs divided into 2 groups:

Group 1: Amniotic membranes were preserved fresh in a mixture of saline and antibiotic in the refrigerator.

Group 2: Amniotic membranes were cryopreserved by frozing in -80$^{o}$C.

3.2. Method of preparation and preservation:

3.2.1. Amniotic membrane harvesting and preparation (Fresh Canine AM):

The placenta was immersed in one liter normal saline 0.9 percent (Al- Mottahedeh Pharma, Egypt) containing 100 U/ml penicillin and 0.2 mg/ml streptomycin (pen & strept®; Noorbrook Netherland) and

0.025 mg/ml amphotericin B after anaesthesia and laparo-hysterotomy of full-term pregnant bitches (amniotic membrane donors according to [21,22,23].
The amniotic membrane was then removed from the chorion by blunt dissection. The epithelial layer was identified by an exterior silk stitch knot that lay on the amniotic membrane's surface. Following careful sterilization, the membranes were serially washed 10 times in sterile Petri-dishes containing 20 ml normal saline and 100 U/ml penicillin, 0.2 mg/ml streptomycin, and 0.025 mg/ml amphotericin. During washing, the separate membranes were gently finger massaged and squeezed to remove the excess blood clots, then maintained in a container containing normal saline with antibiotics and antifungal additives for two hours chilling in a fridge. This preparation with slight modification was according to [21,22,23].

3.2.2. Preservation of Amniotic Membranes for a Long Time (Cryopreserved Canine AM**)**:
The amniotic membranes were transported to the laboratory in a sterile plastic bag in an icebox for prolonged storage over one year. The membrane was cleaned many times with normal saline containing antibiotics and antifungal combination under lamellar flow. The amniotic membranes were molded on a sterile nitrocellulose membrane NC2 (SERVA-Germany; 5 sheets 20x20cm with o.2 m pores size) during immersion. As the molded membrane sheet became adherent to the membrane, it was sliced into 4cm x 5cm pieces with care to avoid exposing the epithelial layer. Each cut portion of the molded membrane sheet was placed in a sterile plastic Petri-dish containing 50% DMEM (Sigma Company's Dulbecco's modified Eagle's medium) or MEM 50% (Minimal essential medium, Sigma Company), both media were in a ratio with glycerol 50% (1:1) with antibiotics and antifungal additives. NuAireUltra-Low Freezer was used to keep the collected membranes at a temperature below freezing (-80°C). After thawing at room temperature, the conserved amniotic membranes were employed as transplants. This preservation with slight modification according to [7,21,23]. The physical characteristics of amniotic membrane were measured according to [24,25].The properties were glistening, tensile strength, thickness and transparency.

## 4. Results

4.1. Physical characters of fresh and frozen amniotic membrane:
Fresh and frozen AM were similar in physical characters such as glistening, tensile strength, thickness, and transparency (fig. 7). The frozen one was turbid during thawing at room temperature and then retain its transparency after about 3 minutes.

4.2. Histology:
Histological findings of the Fresh amniotic membrane revealed the presence of all layer epithelium, basement membrane, compact layer, fibroblast layer, and spongy layer (arrows) in an intact manner resembling natural histological characters of amniotic membrane (Fig. 8a). On the other hand, preserved amniotic membrane showed damaged epithelial cells (Fig. 8b).

## 5. Discussion

The susceptibility of amniotic membrane graft rejection raises the curiosity of regenerative therapy specialists. On the other hand, the amniotic membrane as a source of stem cells and acting as a physical scaffold is related to the methods of preparation and preservation. The amniotic membrane's cell viability was mostly determined by the preservation process's medium composition and keeping temperature [26]. . According to Kruse et al. [7]; Sheta et al. [27] the transplantation of viable amniotic epithelium onto an inflammatory ocular surface could elicit immunological reactions and allow for cell

proliferation from the amniotic membrane. As a result, freezing the amniotic membrane in 50% glycerol, which severely destroys amnion cells, eliminates these drawbacks. As a result, the cryopreserved amniotic membrane is a powerful tool for reconstructing many eye diseases. Cryopreservation, as stated by Lee and Tseng. [21], is regarded as the best method for preservation. Kruse et al. [7] stated that the preparation procedure necessitates the use of a particular preservation approach to protecting the biological qualities of the amniotic membrane. Meryman. [28]; Mazur. [29] and Kruse et al. [7] all concurred that the presence of 50% glycerol functions as a protective reagent.

Moderate finger membrane rubbing is also required to remove blood lodged in the tiny blood vessels of the amniotic membrane in order to avoid rejection according to [7,21,23].

Histologically in the present study, Fresh amniotic membrane revealed presence of all layer epithelium, basement membrane, compact layer, fibroblast layer and spongy layer in intact manner resemble to natural histological characters of amniotic membrane. In the other hand, preserved amniotic membrane showing damaged epithelial cells. These findings approved that of fresh canine AM storage kept its morphological characteristics and epithelial cells viability, while cryopreserved one with glycerol showed that the membrane retained its morphological characteristics although the cellular components had lost their viability as the cells were severely damaged and they were not able to show any signs of viability in vital stain mainly due to epithelial cells rupture during thawing [7,20]. From the obtained data, there were no differences between the physical properties of fresh and preserved amniotic membranes which included glistening, tensile strength, thickness and transparency . The results which agreed to the previously mentioned by Yusof and Hilmy [24].

## 6. Conclusion

From the present study, it could be concluded that the method of fresh amniotic membrane storage retained the morphological characteristics (basement membrane and stromal matrix) and epithelial cells viability which is usefully used as a potent source of stem cells and biological scaffold in regenerative therapy as in allograft. Meanwhile, cryopreserved amniotic membrane held its morphological characteristics, it lost the viability of the epithelial cells so it is recommended to use as a biological scaffold in cases with risk of rejection due to antigenic load of the tissue as in xenograft.

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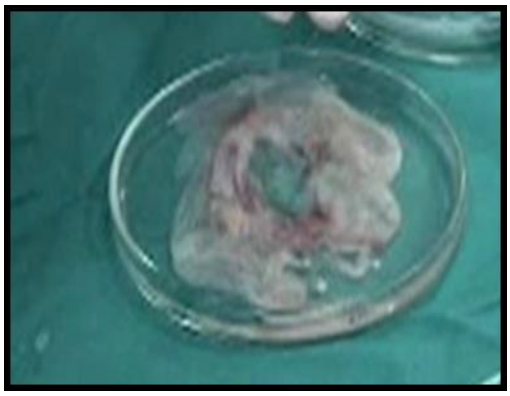

Fig (1): Showing the extracted amniotic Membrane.

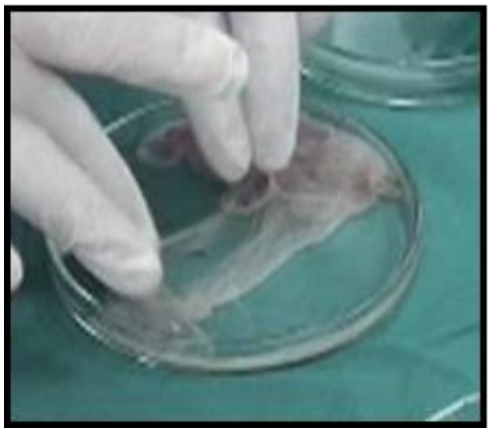

Fig (2): Gently squeeze the minute blood vessel with fingers.

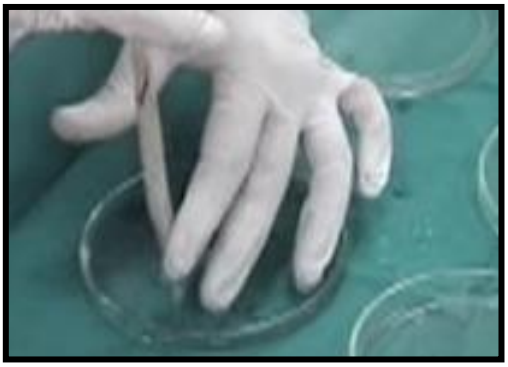

Fig (3): Showing serial washing with NS 0.9% solution.

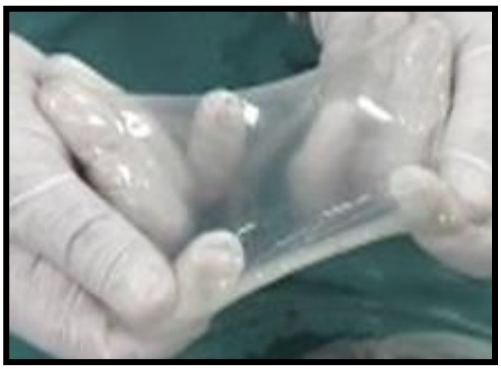

Fig (4): Showing clear amniotic membrane from blood debris.

Fig (5): Showing DMEM, glycerol, and nitrocellulose membrane.


Fig (6): Showing mixing of DMEM and glycerol (1:1).

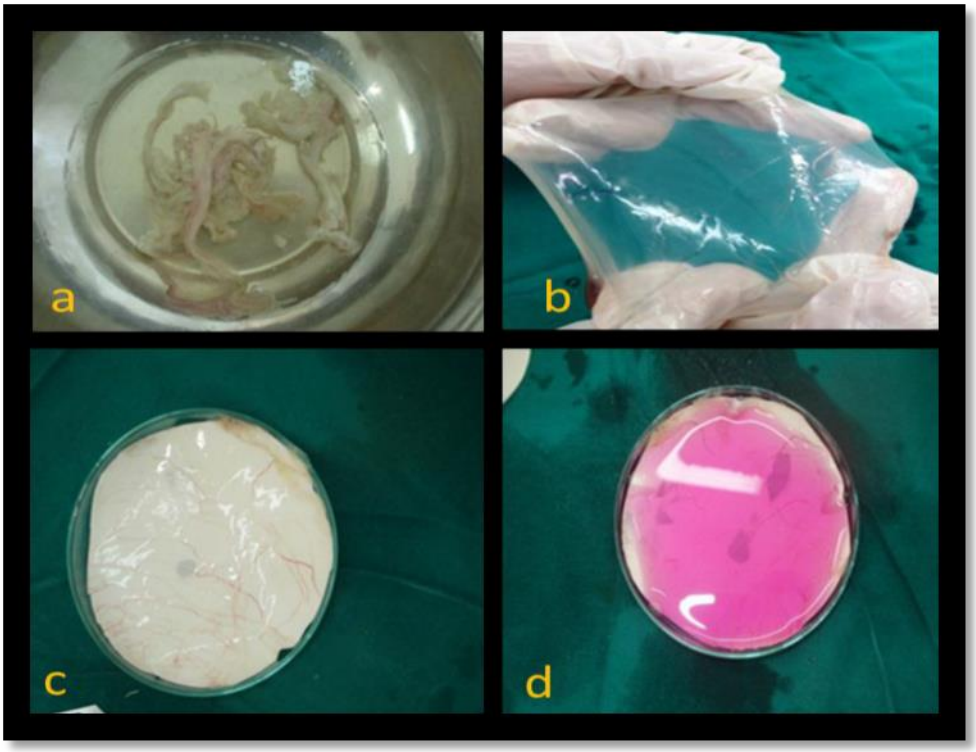


Fig (7): (a and b) showing clinical presentation of canine fresh amniotic membrane
(c and d) showing the clinical presentation of canine frozen amniotic membrane

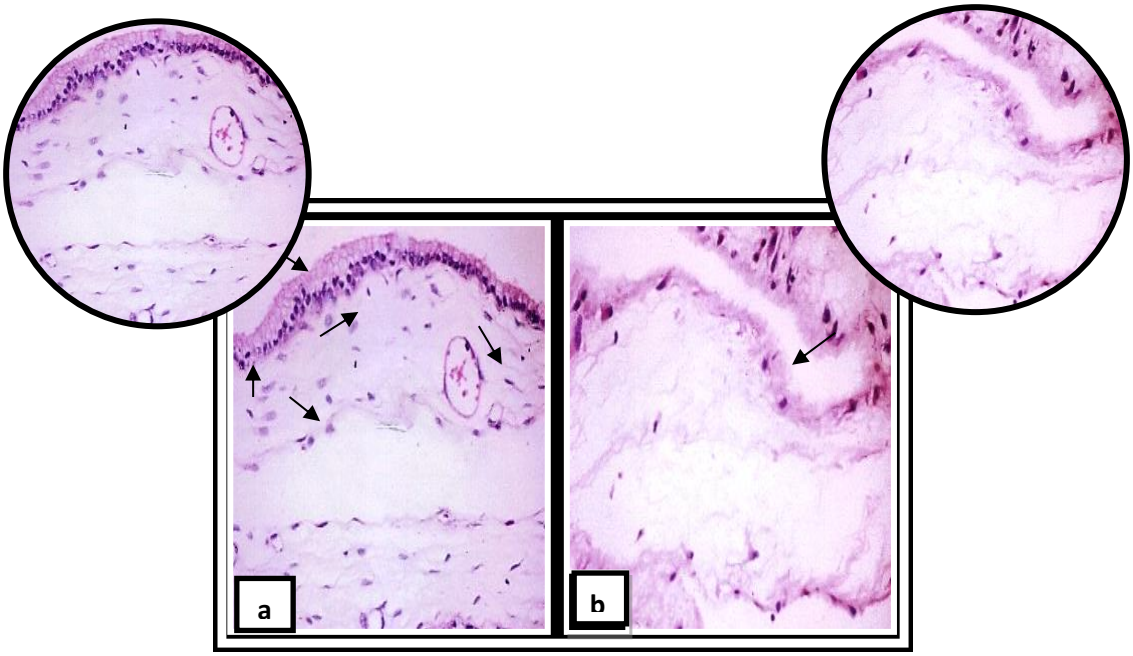


Fig (8): Showing the histology of fresh (a) and cryopreserved (b) amniotic membranes stained with H&E stain.