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Original article

Efficacy of two different antibiotic solutions in preservation of fresh amniotic membrane

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Summary

Introduction. The amniotic membrane is used in transplant surgery, ophthalmology and dermatology. Various methods have been developed to preserve amniotic membrane: hypothermic storage, cryopreservation, lyophilization. Transplantation of fresh amniotic membrane showed low inflammatory response. The efficient antibiotic solutions are carefully chosen for the hypothermic storage of amniotic membranes. The aim of this study was to compare the efficacy of two antibiotic solutions for the hypothermic amniotic membrane preservation and the structure of the amniotic membrane after the preservation process.

Methods. Fifteen amniotic membranes were prepared and hypothermically stored in penicillin solution in BSS (2000 IU/ml) while the remaining fifteen in an antibiotic solution containing: benzylpenicillin (50 µg/ml), gentamicin (100 µg/ml), ciprofloxacin (200 µg/ml) and fluconazole (100 µg/ml). All amniotic membranes were microbiologically tested after preparation and after hypothermic storage for two weeks. Histological analysis of thirty amniotic membranes was performed after the process of preservation.

Results. Fifteen amniotic membranes were sterile after hypothermic preservation in the penicillin solution. Also, fifteen amniotic membranes were sterile after hypothermic preservation in the solution of antibacterial agents (penicillin, gentamicin, ciprofloxacin) and antifungal agent fluconazole. The amniotic membrane had a normal structure and thickness of 35.33±11.03 µm.

Conclusion. Both antibiotic solutions, one that contains only penicillin and one that contains multiple antibacterial agents and fluconazole, provide sterility of fresh hypothermically stored amniotic membranes for two weeks. In the preparation of fresh hypothermically stored amniotic membrane, a solution with multiple antibiotics is preferred. The normal tissue structure of the amniotic membrane was histologically confirmed after the process of preservation.

Keywords: amniotic membrane, preservation, tissue, histological characteristics

Introduction

The placenta is a temporary organ used for the development and growth of the fetus and provides the transfer of the oxygen and nutrients from the mother's organism. It consists of the maternal and the fetal part. Amnion is an extraembryonic membrane that participates in the construction of the fetal part of the placenta. It represents the innermost layer of the placenta and completely envelops

the fetus, forming a cavity filled with amniotic fluid in which the fetus floats. The amnion overlies the chorion and, by its separation from the chorion, an amniotic membrane is produced - AM. AM is a thin, tough, transparent membrane that is avascular and contains no nerves [1, 2]. The thickness of the amniotic membrane is between 20-50 μm and it is composed of three layers: a single epithelial layer, abasement membrane and stroma which is further subdivided into compact, fibroblast and spongy layer [3].

Because of its many beneficial characteristics and unlimited availability, the easy procurement and the low processing costs for therapeutic application, the amniotic membrane represents a valuable biological material used in transplant surgery, ophthalmology, dermatology, plastic surgery, with great potential for use in regenerative medicine for the promotion of nerve regeneration [4-6]. AM shows low or no immunogenicity and promotes wound healing and epithelialization. It is immunologically inert and it is not rejected by the recipient since its cells have only an incomplete HLA antigen. It is bacteriostatic, antiangiogenic, it inhibits scarring and suppresses inflammation. Amniotic membrane, epithelium and stroma contain many growth factors, including epidermal growth factor (EGF), keratocyte growth factor, basic fibroblast growth factor (bFGF), transforming growth factor α (TGFα) and TGFβ, hepatocyte growth factor and nerve growth factor [7, 8, 9]. Epidermal growth factor and keratocyte growth factor are one of the most important growth factors in the promotion of wound healing. TGF-ß has an important role in anti-scarringaction. Anti-inflammatory cytokines released in the amniotic epithelium and stroma, such as asinterleukin-10 and interleukin-1 receptor antagonist, suppress inflammatory processes. Considering that AM avascular tissue and a few anti-angiogenic proteins are identified, AM represents a good tissue for eye transplantation, because anti-angiogenic molecules are essential in ocular tissue and they keep cornea avascular [10].

With the development of the preservation and preparation methods, the amniotic membrane becomes widely used in ophthalmology, burns, plastic surgery, dentistry, and neurosurgery [11].

Various methods have been developed to prepare and preserve the AM: hypothermic preservation, cryopreservation, lyophilization and preservation with glycerol at different temperatures. These methods differentially affect the morphological, biological and physicochemical properties of AM. The AM prepared with cryopreservation method has a higher growth factor level compared to the AM prepared with an additional freezing step that influences great destruction and devitalization of AM tissue. Optimization of an effective and safe method for the preparation and preservation of AM for a specific application is a critical task [12]. When the cryopreserved amniotic membrane from a tissue bank is not available, a fresh amniotic membrane is an alternative. In hospitals where there is the limitation related to costs and availability of the use of cryopreserved amniotic membrane from the tissue bank, the use of a fresh amniotic membrane is an option that provides the same results as the transplantation of cryopreserved amniotic membrane [13, 14]. Transplantation of fresh hypothermically stored amniotic membrane showed low-grade inflammatory response prevention of collagen decomposition of the eye surface. Hypothermic storage has a low destructive effect on the tissue and the concentration of the biologically active substances is very high [13]. Another benefit of a fresh amniotic membrane is that it is stored in an antibiotic solution. Antibiotics can subsequently be released from the membrane [15, 16]. The AM soaked with an antibiotic solution for a period of 3h at 4°C caused high drug entrapment in AM. Studies showed that 3h pretreatment might be sufficient to fill up the membrane with drug formulations. Therefore, AM may be an excellent drug carrier and the drug reservoir function of AM was demonstrated with moxifloxacin, ofloxacin, and cefazolin. It is reported earlier that AM transplantation is useful adjunctive treatment to cefazolin for staphylococcus aureus keratitis. The previous research showed that AM could release antibiotics for an extended period of 7 weeks [16].

In previous studies, the authors used different antibiotic cocktail solutions for the preparation and hypothermic storage of the fresh amniotic membrane [17, 18]. The antibacterial agents are carefully chosen for the combination of antibi-

otics that provides effective treatment against bacteria commonly found to contaminate the tissue. The aim of the study was to compare the efficacy of two different antibiotic solutions for the hypothermic amniotic membrane preservation and the structure of the amniotic membrane after the preservation process.

Methods

Thirty amniotic membranes were taken from donors (pregnant women), after the completion of pregnancy performed by the cesarean section. Donors were healthy pregnant women with no history of sexually transmitted diseases and without endometritis, toxemia, premature rupture of membranes, signs of inflammation of the fetal sheath, and amnion stained with meconium. The amniotic membrane was separated from the placenta, which was discarded after delivery and represented a medical waste. Considering previously described steps, the AM was easy to obtain without harming mothers or babies. However, legally it was still the possession of the mother, so the consent letter concerning the AM use and analysis was obtained from the mother. Before obtaining the amniotic membrane from the placenta, it was necessary to obtain the written consent of the tissue donor (pregnant woman) for performing appropriate serological tests. Consent was obtained for blood tests for human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV). Preparation, preservation and use of the human amniotic membrane were approved by the Ethics Committee of the UCC RS Banja Luka.

The amniotic membrane was obtained from the placenta after elective cesarean sections. All donors were serologically tested for the presence of HIV, HBV and HCV. To shorten and avoid the window period, i.e. the period from a possible donor infection to its seropositivity, the presence of the viral DNA and RNA was analyzed by the PCR method. Also, the presence of antibodies against HBc antigens, HBs antigens, HCV antigens, and HIV were determined by serological tests. Before the cesarean section, the ophthalmologist had to inform the obstetrician about taking AM. In the operating gynecological room, during the cesarean section, the obstetrician surgeon had to put the placenta in a sterile container. Then the sterile container with the placenta was taken by an ophthalmologist, who transferred it to a table covered with a sterile compress. Previously, sterile instruments were placed on a sterile table. Also, an infusion system coupled with an antibiotic solution in a balanced salt solution - BSS, and sterile containers in which AM would be flushed and prepared. The whole procedure of obtaining and preparing the amniotic membrane was performed in the sterile operating room of the Gynecology Clinic. The ophthalmologist had to bedress in a surgical uniform with a cap and mask on his head. After surgical washing, he entered the operating room, where he wore sterile coat and gloves. Before the cesarean section, the table with the necessary instruments, containers, and solutions was prepared.

The placenta had to be oriented in a way that the fetal side, which was covered by the amnion, faced upwards. After that, blood clots were removed from the amnion with surgical forceps and sterile pads soaked into the solution of antibiotics in BSS. The separation of the amnion from the chorion was done by blunt dissection (Figure 1). Then, ophthalmologist washed the separated AM with an antibiotic solution in a BSS. Fifteen amniotic membranes were prepared using

Figure 1. Obtaining the amniotic membrane

an antibiotic solution in BSS containing benzyl penicillin (50 µg/ml), gentamicin (100 µg/ml), ciprofloxacin (200 µg/ml) and fluconazole (100 µg/ml). The same antibiotic solution was used for the preservation of the amniotic membrane. Another fifteen amniotic membranes were then prepared using a solution of penicillin in a balanced salt solution at a concentration of 2000 IU/ml. These fifteen amniotic membranes were also stored in the same solution. After rinsing, the amnion was placed in a sterile glass jar containing the same antibiotic solution in the balanced salt solution (BSS), with which the amnion was rinsed. The next important step was rapid transport in a hand-held refrigerator to the laminar chamber. The amnion was further processed in the laminar chamber and was stretched onto nitrocellulose paper, which was the carrier for the amniotic membrane (Figure 2). After rinsing again, the amnion was placed onto filter paper and then it was cut into small size pieces with dimension about 4x4 cm or 2x2 cm. Amniotic membranes were individually packaged in a sterile, properly labeled, plastic containers. A piece of a randomly selected portion of the amnion was used for microbiological testing. Furthermore, the antibiotic solution that was used for the preparation and storage of the fresh amniotic membrane was microbiologically tested. Then the amniotic membrane was stored hypothermically at +4°C in the refrigerator. In this way processed and stored amniotic membranes, after obtaining negative serological and microbiological results, were released and used in clinical practice. The maximum storage time at +4 °C was two weeks. The microbiological safety of the amniotic membranes was monitored after two weeks.

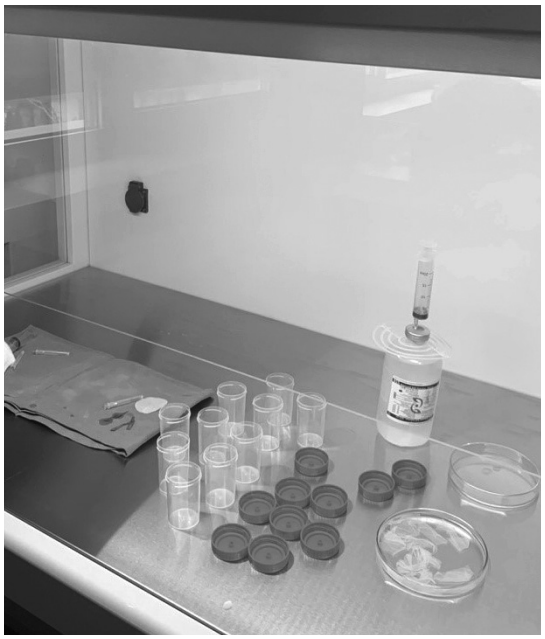

Figure 2. The material required for the preparation of amniotic membranes

The thirty samples of amniotic membrane and twenty samples of the placenta were histologically processed for paraffin-embedded sections at the Department for Histology and Embryology at the Faculty of Medicine in Banja Luka. The samples were oriented so that the cut was made perpendicular to the epithelium. The 5 µm thick tissue sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (HE) and periodic acid-Schiff stain (PAS). Histological analysis and morphometry were performed using a Leica DM 6000 B microscope and Image Analysis LAS V4.3 software. The results were analyzed by methods of descriptive statistics.

Results

In the operating room under sterile conditions, the amnion was separated from the thirty placentae. After the elective cesarean section, the placenta was placed in a sterile vessel. Discoid-shaped placentae was oriented in such a way that the fetal side faced upwards and the maternal side downwards. The fetal side was smooth, shiny and transparent because it was covered with transparent amnion. In most cases, the umbilical cord was attached near the central part of the fetal side.

The placenta consisted of two opposite plates: chorionic and basal. They limit the space in which the chorionic villiand maternal blood were placed. The fetal side of the placenta was built by a chorionic plate. Looking from the fetal side to the maternal side, in the chorionic plate, the following layers were differentiated: the amniotic epithelium, the amniotic mesenchyme, the chorionic mesenchyme, and the trophoblast (Figure 3).

After preliminary preparation of the placenta and removal of the coagulums of blood, the amnion has been separated from the placenta by blunt dissection with sterile instruments. Amni-

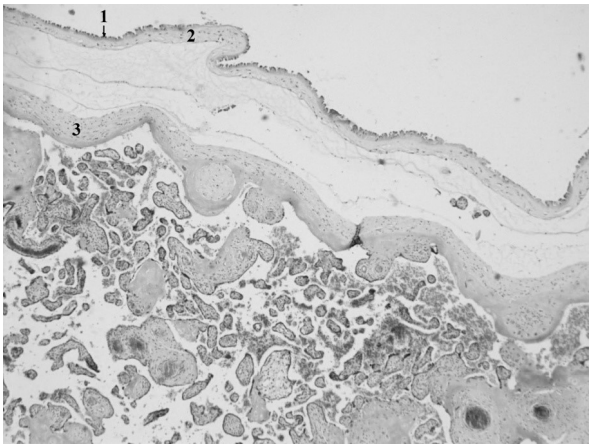

Figure 3. The Chorionic plate: 1. amniotic epithelium (arrow), 2. amniotic mesenchyme 3. chorionic mesenchyme (HE,x100)

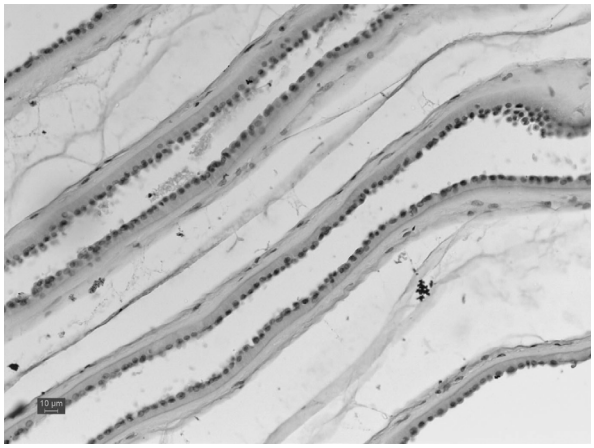

Figure 4. The Amniotic membrane (HE,x200)

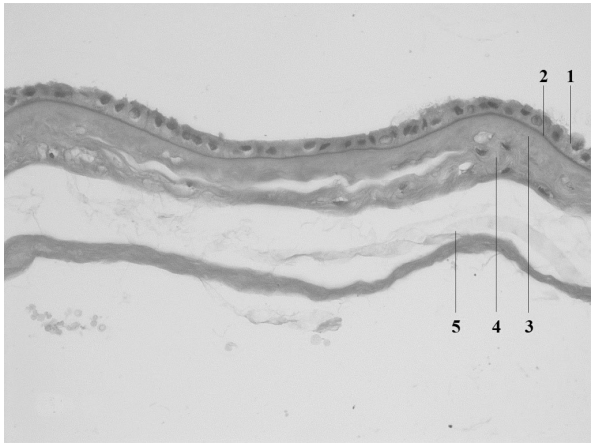

Figure 5. The amniotic membrane: 1. amniotic epithelium, 2. basal membrane, 3. compact acellular layer, 4. fibroblast layer, 5. spongy layer (PAS,x400)

on consisted of the epithelium, basement membrane, and stroma (Figure 4). Three sublayers were differentiated in the stroma: the compact layer, the fibroblast layer, and the spongy layer (Figure 5). The amniotic epithelium was built up of a single cuboid cell layer with a centrally located single nucleus. The amniotic epithelium rested on a basement membrane that was strongly PAS-positive. Beneath the basement membrane was an acellular compact layer. The compact layer overlaid the fibroblast layer that was the thickest layer of the amnion. This layer was made up of sparsely distributed fibroblasts. The deepest layer of the amniotic membrane was the spongy layer (Figure 5).

The amniotic membrane, the inner layer of the chorionic plate, had an average thickness of 35.33 ± 11.03 µm. The minimum thickness of the amniotic membrane was 17.99 µm and the maximum thickness of the amniotic membrane was 50 µm.

Serological tests showed that all donors, the pregnant women, were healthy. In the blood of thirty donors, the pregnant women, the PCR method did not detect HBV, HCV and HIV. With the method of obtaining and preparation of the fresh amniotic membrane, we got sterile amniotic membrane stretched on a nitrocellulose filter paper. From all thirty microbiologically tested samples

Table 1. Results of microbiological tests

	Results of microbiological tests	
	After processing	After two weeks of hypothermic storage
The samples of amniotic membranes processed and stored with an antibiotic solution in BSS (benzylpenicillin 50 µg/ml, gentamycin 100 µg/ml, ciprofloxacin 200µg/ml, fluconazole 100 µg/ml)	All sterile (100%)	All sterile (100%)
The samples of amniotic membranes processed and stored with penicillin solution in BSS (penicillin 2000 IU/ml)	All sterile (100%)	All sterile (100%)

of amniotic membranes, which were taken after amnion processing and before its packaging in sterile containers, bacteria and fungi were not isolated by microbiological testing (Table 1). Fifteen fresh amniotic membranes, which were washed during processing in the solution of penicillin in a balanced salt solution (BSS) at a concentration of 2000 IU/ml, showed sterility after processing. Also, fifteen fresh amniotic membranes showed sterility after preparation in the solution of antibiotics in BSS, containing benzyl penicillin (50 µg/ml), gentamicin (100 µg/ml), ciprofloxacin (200 µg/ml), fluconazole (100 µg/ml) (Table 1). By checking the microbiological suitability of the two solutions for rinsing and storage, the solution of antibiotic cocktail in BSS and the penicillin solution in BSS at a concentration of 2000 IU/ml, the bacteria and fungi were not isolated.

The obtained results showed that all amniotic membranes were sterile after two weeks of hypothermic storage at +4 °C, stored in the same antibiotic solution with which they were processed (Table 1).

Discussion

Blunt dissection easily separated the amniotic membrane on the fetal side of the placenta. The thickness of the amniotic membrane was 35.33 ± 11.03 µm. It was composed of an amniotic epithelium, a basal lamina, a compact avascular layer, a fibroblast layer and a spongy layer. In the previous studies, the amniotic membrane had been described as a thin, tough, transparent membrane, its thickness being between 20-50 µm, and was composed of three layers: a single epithelial layer, a thick basement membrane, and stroma subdivided into a compact, fibroblast and spongy layer [1, 2].

In this study, thirty sterile amniotic membranes stretched on nitrocellulose filter paper were obrained from the thirty placentae. With microbiological testing of the amniotic membrane samples, after processing and before packaging of AM into sterile containers, no bacteria and fungi were isolated. Fifteen amniotic membranes which were rinsed during processing with penicillin solution in a BSS were found sterile after processing. Also, these amniotic membranes were sterile after two weeks of hypothermic storage in the same solution and at a temperature of +4 °C. Fifteen fresh amniotic membranes showed sterility after preparation and preservation in the solution of antibacterial agents (benzylpenicillin, gentamicin, ciprofloxacin) and antifungal agent fluconazole.

In previous studies, Ganatra and Durrani used antibiotic solution, containing 2000 IU crystalline penicillin/ml NaCl 0.9%, for decontamination during the processing of the fresh amniotic membranes and their hypothermic storage at +4°C temperature. In this study, microbiological tests of all amniotic membranes, after their preparation, showed sterility. Also, the amniotic membrane was sterile after hypothermic storage at +4°C temperature, during two weeks, in an antibiotic solution containing 2000 IU crystalline penicillin/ml NaCl 0,9% [17, 18]. Fifteen amniotic membranes, after processing with the antibiotic solution in BSS containing benzylpenicillin (50 µg/ml), gentamicin (100 µg/ml), ciprofloxacin (200 µg/ml), and fluconazole (100 µg/ml) were sterile. Also, the amniotic membrane was sterile after hypothermic storage at 4°C temperature for two weeks in the same solution of antibiotics. Antibiotic cocktail for sterilizing tissue contained antibacterial and antifungal agents that had to be present in amounts effective to inhibit fungal and bacterial growth. Each of the agents was present in a sufficient concentration so that the cocktail inhibited yeast and bacterial growth but did not substantially decrease the viability of the tissue being decontaminated [19, 20]. The agents that were present in the cocktail were cidal for yeasts and bacteria frequently isolated from tissue. The yeast and bacterial growth were not detectable by standard microbiological assays, after the tissue was treated with such a cocktail. The broad-spectrum antibacterial agents from two or more families were preferred in an antibiotic cocktail. Preferably the plurality of antibacterial agents was chosen from the following families: cephalosporins, glycopeptides, aminoglycosides, lincosamides, beta-lactams and rifamycin [19]. Antifungal agents used in cocktails were amphotericinB or fluconazole. A concentration of fluconazole from about 50 g/ml to about 100 g/ml was preferred in antibiotic cocktails.

Waziri used a balanced salt solution (BSS) containing gentamycin antibiotic for the preparation of fresh amniotic membrane that was used in ophthalmology [21]. Abbasi and coauthors used an antibiotic solution containing streptomycin 50 µg/ml, gentamicin 100 µg/ml, benzylpenicillin 50 µg/ml [22] for the preparation of the amniotic membrane. For the preparation of amniotic membrane and its storage at +4°C for two weeks, Salman and coworkers used antibiotic cocktail containing 50 µg/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml, neomycin and 2.5 µg/ml amphotericin B in a balanced salt solution [23]. Madhavan and coauthors used the preservative medium 1:1 (vol/vol) ratio of sterile glycerol (sterilized by autoclave) and EMEM with 3.3% L-glutamine, 25 µg/ml gentamicin, 50 units/ml penicillin, 100 µg/ml ciprofloxacin and 0.5 mg/ml amphotericin B [10]. Dekaris and Gabrić extensively rinsed the amniotic membrane with BSS solution with antibiotic supplementation (penicillin 50 µg/ml, streptomycin 50 µg/ml or ciprofloxacin 200 µg/ml, amphotericin B 2.5 µg/ml, neomycin 100 µg/ml) during preparation [24]. Cirman and coauthors rinsed amniotic membrane with BSS solution with antibiotic supplementation (penicillin 50 µg/ml, streptomycin 50 µg/ml or ciprofloxacin 200 µg/ml, amphotericin B 2.5 µg/ml, neomycin 100 µg/ml) [5].

Although both antibiotic solutions provided sterility for the amniotic membrane, one that contained only penicillin and one that contained multiple antibacterial agents and fluconazole, a more preferred solution for the hypothermic storage of the amniotic membrane was the one containing multiple antibacterial agents and an antifungal agent. The method described in this study provided a fresh hypothermically stored amniotic membrane that can be used for all indications and conditions in clinical practice for which cryopreserved amniotic membrane was recommended. Lacking cryopreserved amniotic membrane from a tissue bank, the transplantation of the fresh hypothermically preserved amniotic membrane was a good and recommended alternative.

Conclusion

Both antibiotic solutions, one that contains only penicillin and one that contains multiple antibacterial agents and fluconazole, have provided sterility of fresh hypothermically stored amniotic membranes for two weeks. The solution that contains multiple antibacterial agents and fluconazole is more preferred for the preparation and hypothermic storage of the amniotic membrane. Histological analysis has confirmed the normal tissue structure of the amniotic membrane after the process of hypothermic preservation. The method used for the hypothermic storage of the amniotic membrane represents a safe method that provides sterile amniotic membranes suitable for the application. The hypothermic storage of amniotic membrane ensures the accessibility of the amniotic membrane when the cryopreserved amniotic membrane from the tissue bank is not available.

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Ethical approval. The Ethics Committee of the University Clinical Center of the Republic Srpska in Banja Luka approved the study and informed consent was obtained from all individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Efikasnost dva različita antibiotska rastvora u prezervaciji svježe amnionske membrane

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Uvod. Amnionska membrana koristi se u transplantacijskoj hirurgiji, oftalmologiji, dermatologiji. Za prezervaciju amnionske membrane su razvijene različite metode: hipotermno čuvanje, krioprezervacija, liofilizacija. Transplantacija svježe hipotermno čuvane amnionske membrane izaziva inflamatorni odgovor niskog intenziteta. Za pripremu i hipotermno čuvanje amnionske membrane bira se antibiotski rastvor koji će efikasno djelovati protiv mikroorganizama koji obično kontaminiraju tkivo. Cilj ove studije bio je da se uporedi efikasnost dva različita antibiotska rastvora za prezervaciju svježe amnionske membrane i utvrdi histološka struktura amnionske membrane nakon postupka prezervacije.

Metode. Petnaest svježih amnionskih membrana je hipotermno prezervirano u rastvoru penicilina u BSS (koncentracija 2000 IU/ml BSS), a petnaest u rastvoru antibiotika u BSS koji sadrži benzilpenicilin (50 µg/ml), gentamicin (100 µg/ml), ciprofloksacin (200 µg/ml) i flukonazol (100 µg/ml). Sve amnionske membrane su nakon pripreme i nakon hipotermnog čuvanja, tokom dvije sedmice, mikrobiološki testirane. Histološka analiza trideset amnionskih membrana nakon procesa prezervacije je urađena.

Rezultati. Petnaest amnionskih membrana je bilo sterilno nakon hipotermne prezervacije u rastvoru penicilina. Takođe, petnaest amnionskih membrana je bilo sterilno nakon hipotermne prezervacije u rastvoru antibakterijskih agenasa (penicilin, gentamicin, ciprofloksacin) i flukonazola. Histološkom analizom amnionskih membrana nakon procesa prezervacije je utvrđena očuvana histološka struktura. Prosječna debljina amnionske membrane je bila 35,33±11,03 µm.

Zaključak. Oba antibiotska rastvora, onaj koji sadrži samo penicilin i onaj koji sadrži više antibakterijskih agenasa i flukonazol, obezbjeđuju sterilnost svježih hipotermno čuvanih amnionskih membrana tokom dvije nedjelje. U pripremi svježe hipotermno čuvane amnionske membrane prednost ima rastvor sa više antibiotika. Normalna struktura tkiva amnionske membrane je histološki potvrđena nakon procesa prezervacije.

Ključne riječi: amnionska membrana, prezervacija tkiva, histološke karakteristike