eOftalmo

NARRATIVE LITERATURE REVIEW

Protocol for procuring, processing, and storing human amniotic membrane for ophthalmic use

Protocolo de captação, processamento e armazenamento de membrana amniótica humana para uso oftalmológico

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KEYWORDS:

Protocols; Tissue banks; Extraembryonic membranes; Tissue and organ procurement; Wounds and injuries.

ABSTRACT

Human amniotic membranes have great therapeutic potential owing to their anti-inflammatory, antimicrobial, and antiangiogenic characteristics. In ophthalmology, their use is well established for treating acute and chronic lesions of the ocular surface, such as persistent corneal defects and descemetocele, and even in elective surgeries such as pterygium excision. The process of obtaining amniotic membranes for ophthalmic use involves several steps, including clinically screening the donor, retrieving the placenta along with the amniotic and chorionic membranes, and processing, storing, and monitoring the amniotic membrane quality. Thus, it is necessary to develop a protocol to serve as a guideline for producing amniotic membrane grafts, preserving their therapeutic characteristics and reducing the risks inherent to their use in recipient patients. This study presents a standard operating protocol for procuring, preparing, and preserving amniotic membranes for ophthalmic use to enable the safe reproducibility of this process.

PALAVRAS-CHAVE:

Protocolos, Bancos de Tecidos; Membranas Extraembrionárias; Obtenção de Tecidos e Órgãos; Ferimentos e Lesões.

RESUMO

A membrana amniótica humana possui grande potencial terapêutico por suas características anti-inflamatórias, antimicrobianas e antiangiogênicas. Na Oftalmologia, seu uso está consolidado para o tratamento de lesões agudas e crônicas da superfície ocular, como defeitos corneanos persistentes e descemetocele, e até mesmo em cirurgias eletivas, como a exérese de pterígio. O processo de obtenção da membrana amniótica para uso oftalmológico possui diversas etapas, que incluem a triagem clínica da doadora, captação da placenta com as membranas amniótica e coriônica, processamento, armazenamento e o monitoramento da qualidade da membrana amniótica. Assim, faz-se necessário o desenvolvimento de um protocolo a servir como orientação para a produção de enxertos de membrana amniótica preservando suas características terapêuticas e reduzindo os riscos inerentes ao seu uso em pacientes receptores. Apresentamos neste artigo um protocolo operacional padrão para captação, preparo e preservação da membrana amniótica para uso oftalmológico, de modo a possibilitar a reprodutibilidade segura deste processo.

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INTRODUCTION

The placenta is an ephemeral maternal-fetal organ with several functions, including transferring nutrients from mother to fetus and eliminating fetal metabolites. Four different membrane structures are involved in the placental development of human beings: chorion, amnion, yolk sac, and allantois[1]. During fetal development, the amnion or amniotic membrane (AM) protects the fetus against mechanical stress and dehydration. In addition, AM has other important properties such as anti-inflammatory, antimicrobial, and antiangiogenic activities[2]. In addition, AM exhibits reduced immunogenicity, contributing to its applicability as a graft.

AM is between 0.02 and 0.5 mm thick and comprises five distinct layers: epithelium, basal membrane, compact layer, fibroblast layer, and spongy layer (Figure 1)[3]. It has been used for therapeutic purposes for the first time in 1910; however, it was only 30 years later that its use was described in ophthalmology for treating symblepharon[2]. Currently, there are studies suggesting benefits of using AM in several other ophthalmologic problems, such as burns, Stevens–Johnson syndrome, toxic epidermal necrolysis, corneal perforations and thinning (e.g., descemetocele, neurotrophic ulcers, and epithelial defects), bullous keratopathy, band keratopathy, limbal stem cell deficiency, conjunctival reconstruction, scleritis, grafting for pterygium, ocular surface tumors, and fistulizing surgeries.

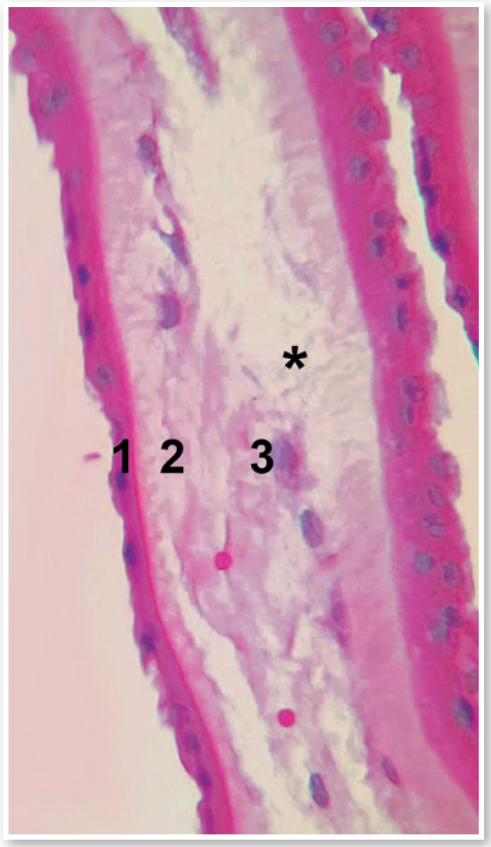


Figure 1. Histological section of an amniotic membrane sample (hematoxylin-eosin, 400× magnification) showing the epithelium with the basal membrane (1), compact layer (2), fibroblast layer (3), and the space where the spongy layer would be located (*). (Figure kindly provided by Prof. Renata Scarpat Careta.)

To use AM for ophthalmologic treatment, it is necessary to perform preparation and storage processes for to decontaminating the material and promoting an environment as inert as possible wherein the tissue can be preserved and its properties can be maintained. There are different preservation methods, including dehydration, freeze-drying, chemical crosslinking, and cryopreservation, which can interfere with the tissue structure of AM and influence its degeneration process and treatment success[4]. A recent study reported that cryopreserved AM is comparable with fresh AM with respect to structural integrity and retention of biochemical components essential for biological functions, indicating that cryopreservation offers a safe and effective means of preservation[5]. Considering the importance of establishing standardized methods for obtaining AM ready for use in ophthalmic procedures, a standardized operational protocol was developed that establishes flows and methods for procuring, processing, and storing AM.

METHODS

National regulations and international recommendations for using human AM in ophthalmology as well as procuring, preparing, and storing this tissue were reviewed from scientific articles published in PubMed, SciELO, and LILACS bibliography databases in addition to the current Brazilian legislation. Articles published till October 31, 2022 were included, using the following keywords in Portuguese and English: "amniotic membrane," "amnion," "transplantation," "preservation," "tissue bank," and "eye bank." Based on these data, an operational protocol was developed to ensure compliance with good clinical, therapeutic, and organizational practices.

RESULTS

Duties, attributions, and responsibilities

The process of procuring and preparing AM tissue to be suitable for use is multidisciplinary and involves the medical and nursing teams of the ophthalmology clinic's eye bank, obstetrics and gynecology clinic, and pathology and microbiology laboratories, as shown in the flowchart described in Figure 2 and detailed below.

Steps

1. Screening of potential donors by the eye bank team of the ophthalmology clinic

1.1. Identify a possible donor: puerperal women aged >18 years admitted to the obstetric ward (meeting the inclusion criteria) who do not meet any of the exclusion criteria. According to the Technical Regulation of the Brazilian National Transplant System, Ordinance No. 2600 of October 28, 2009[6–9] and the criteria of the Eye Bank of the Cassiano Antônio Moraes University Hospital of the Federal University of Espírito Santo, Vitória, Brazil, the absolute contraindications to a candidate for donation were defined as follows:

a) Active sepsis;
b) Active endocarditis (bacterial or fungal);
c) Active disseminated lymphomas;
d) Leukemias;
e) Clinical or laboratory evidence of infection caused by the human immunodeficiency virus (HIV) or hepatitis B or C;
f) Risk of transmission of diseases caused by prions (such as Creutzfeldt–Jakob disease), neurological diseases of viral or undetermined etiology, subacute sclerosing panencephalitis, active viral encephalitis, encephalitis of unknown origin, progressive encephalopathy, or progressive multifocal leukoencephalopathy;
g) Rabies;
h) Congenital rubella;
i) Reye's syndrome;
j) Meningitis;
k) Colonization by multidrug-resistant bacteria;
l) Pesticide poisoning;
m) Leptospirosis;
n) Dengue fever in the febrile or critical phase;
o) Malaria;
p) Herpetic encephalitis;
q) Cancer in a location more likely to metastasize (breast, lung, liver, pancreas, lymphomas, leukemias, and cutaneous melanomas);
r) The following active diseases: cancer of the uterus, cervix, or vaginal canal; endometriosis; endometritis; pelvic inflammatory disease; and intrauterine or vaginal canal infection with the human papillomavirus (HPV).

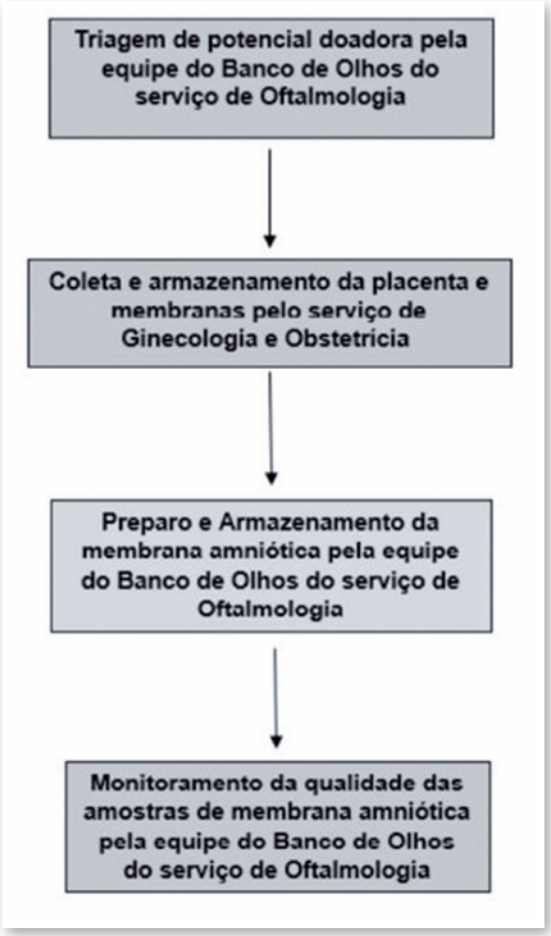


Figure 2. Flowchart of amniotic membrane procurement, preparation, and storage.

1.2 Approach and reception of the pregnant woman for the placenta donation process.

This is a vital step and the interview needs to be conducted responsibly, respectfully, and carefully, especially because the questionnaire presented to the pregnant woman includes personal and intimate questions about their behavior, and therefore, requires a comfortable environment to be answered.

Deliver the Placental Tissue Donation Term (Figure 3), collecting signatures in all three copies. The first copy will be attached to the medical record, the second copy will be attached to the donation record

TERMO DE DOAÇÃO DE TECIDO PLACENTÁRIO

Pelo presente instrumento, EU,_____, natural de _____, RG _____, emitido por:_____, CPF _____, residente e domiciliado à _____, bairro _____, na cidade de _____, estado de_____, CEP _____, telefone _____, na qualidade de _____ (doador, cônjuge, ascendente ou descendente), responsável pelo paciente _____, natural de _____, data de nascimento____/____/ ____, RG nº: _____, CPF: _____, residente e domiciliado à __ _____ filiação _____, autorizo, por minha inteira responsabilidade e por livre e espontânea vontade, o procedimento de retirada de membrana amniótica e amostra sanguínea para realização de exames sorológicos, para fins de transplantes, objetivos terapêuticos e fins científicos, em conformidade com as disposições da Lei nº 9.434 de 04/02/1997, Lei nº 10.211 de 23/03/2001 e o Decreto nº 2.2.68 de 30/06/1997 e toda a legislação pertinente. Fica ressalvado que a utilização para fins científicos somente será permitida depois de esgotadas as possibilidades de utilização para transplante, conforme a Portaria GM nº 263 de 31/03/1999. As informações deste termo são de total responsabilidade do declarante, inclusive o grau de parentesco com o doador. Enfatizo que esta autorização é motivada apenas por razões humanitárias, sem expectativa de receber nenhuma forma de compensação.

_____, _____ de _____ de _____

Assinatura do Responsável pela entrevista familiar

Assinatura da doadora ou responsável

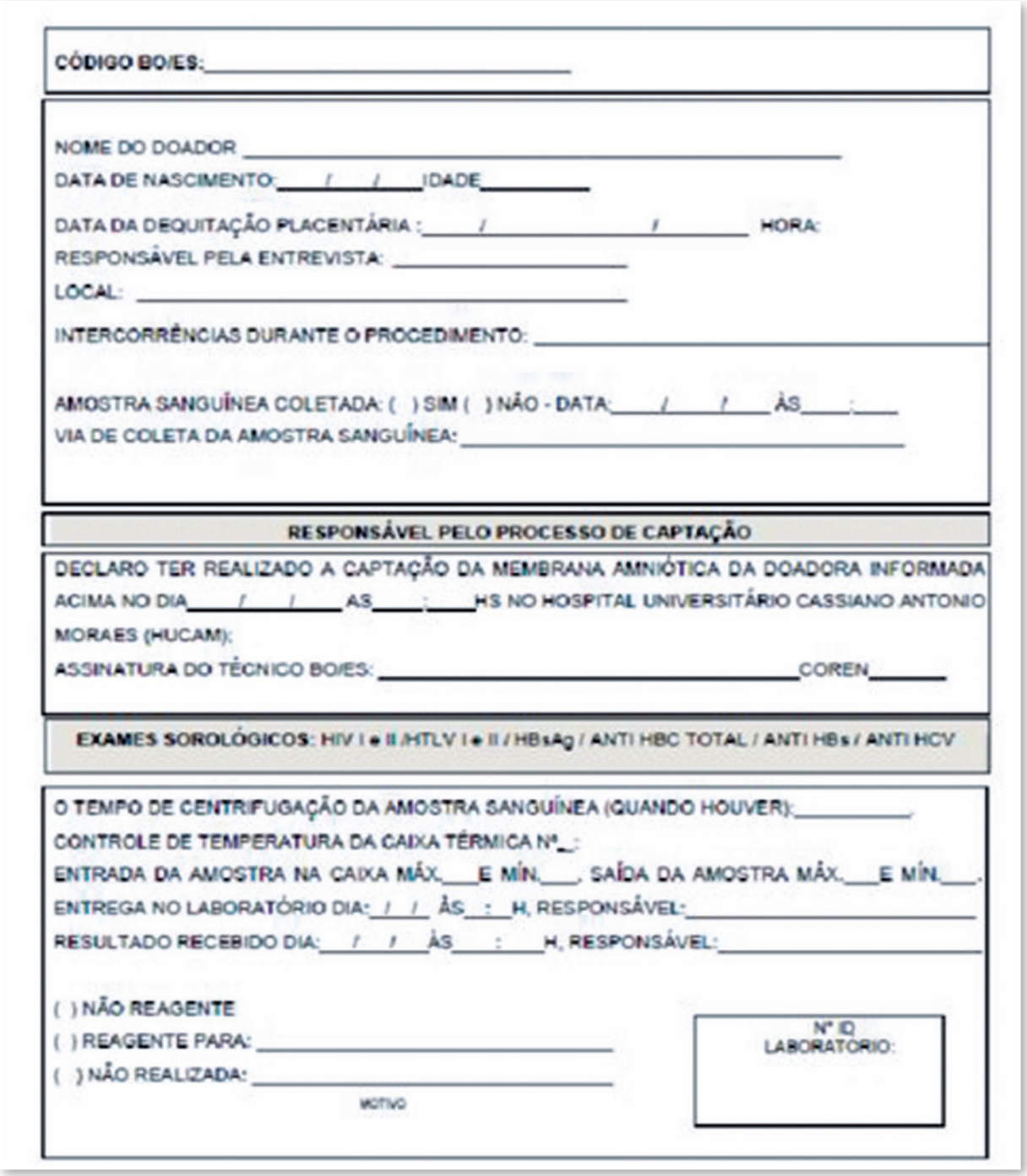
Figure 3. Term of placental tissue donation.

at the eye bank, and the third copy will be provided to the potential donor. Refusal to sign the Placental Tissue Donation Term will be considered an exclusion criterion.

1.3. Identify the initial data of the AM donor (Figure 4), including name, age, time of placental expulsion, medical record number, and family members present;

1.4. Fill out the clinical screening form (Figure 5). Failure to meet all mandatory criteria will make it impossible to use the donated tissue, and it must be disposed of in an appropriate place:

a) Delivery <24 h before the day the graft is made (mandatory criterion);
b) No history of alcohol, tobacco, or drug abuse (mandatory criterion);



CÓDIGO BO/ES:_____

NOME DO DOADOR _____

DATA DE NASCIMENTO:___/___/___IDADE_____

DATA DA DEQUITAÇÃO PLACENTÁRIA :___/_____/_____ HORA:

RESPONSÁVEL PELA ENTREVISTA: _____

LOCAL: _____

INTERCORRÊNCIAS DURANTE O PROCEDIMENTO: _____

AMOSTRA SANGUÍNEA COLETADA: ( ) SIM ( ) NÃO - DATA:___/___/___ÀS___:___

VIA DE COLETA DA AMOSTRA SANGUÍNEA: _____

RESPONSÁVEL PELO PROCESSO DE CAPTAÇÃO

DECLARO TER REALIZADO A CAPTAÇÃO DA MEMBRANA AMNIÓTICA DA DOADORA INFORMADA ACIMA NO DIA___/___/___AS___:___HS NO HOSPITAL UNIVERSITÁRIO CASSIANO ANTONIO MORAES (HUCAM):

ASSINATURA DO TÉCNICO BO/ES: _____COREN_____

EXAMES SOROLÓGICOS: HIV I e II /HTLV I e II / HBsAg / ANTI HBC TOTAL / ANTI HBs / ANTI HCV

O TEMPO DE CENTRIFUGAÇÃO DA AMOSTRA SANGUÍNEA (QUANDO HOUVER):_____

CONTROLE DE TEMPERATURA DA CAIXA TÉRMICA Nº_:

ENTRADA DA AMOSTRA NA CAIXA MÁX.___E MÍN.___. SAÍDA DA AMOSTRA MÁX.___E MÍN.___.

ENTREGA NO LABORATÓRIO DIA:___/___/___ ÀS___:___H, RESPONSÁVEL:_____

RESULTADO RECEBIDO DIA:___/___/___ÀS___:___H, RESPONSÁVEL:_____

( ) NÃO REAGENTE

( ) REAGENTE PARA: _____

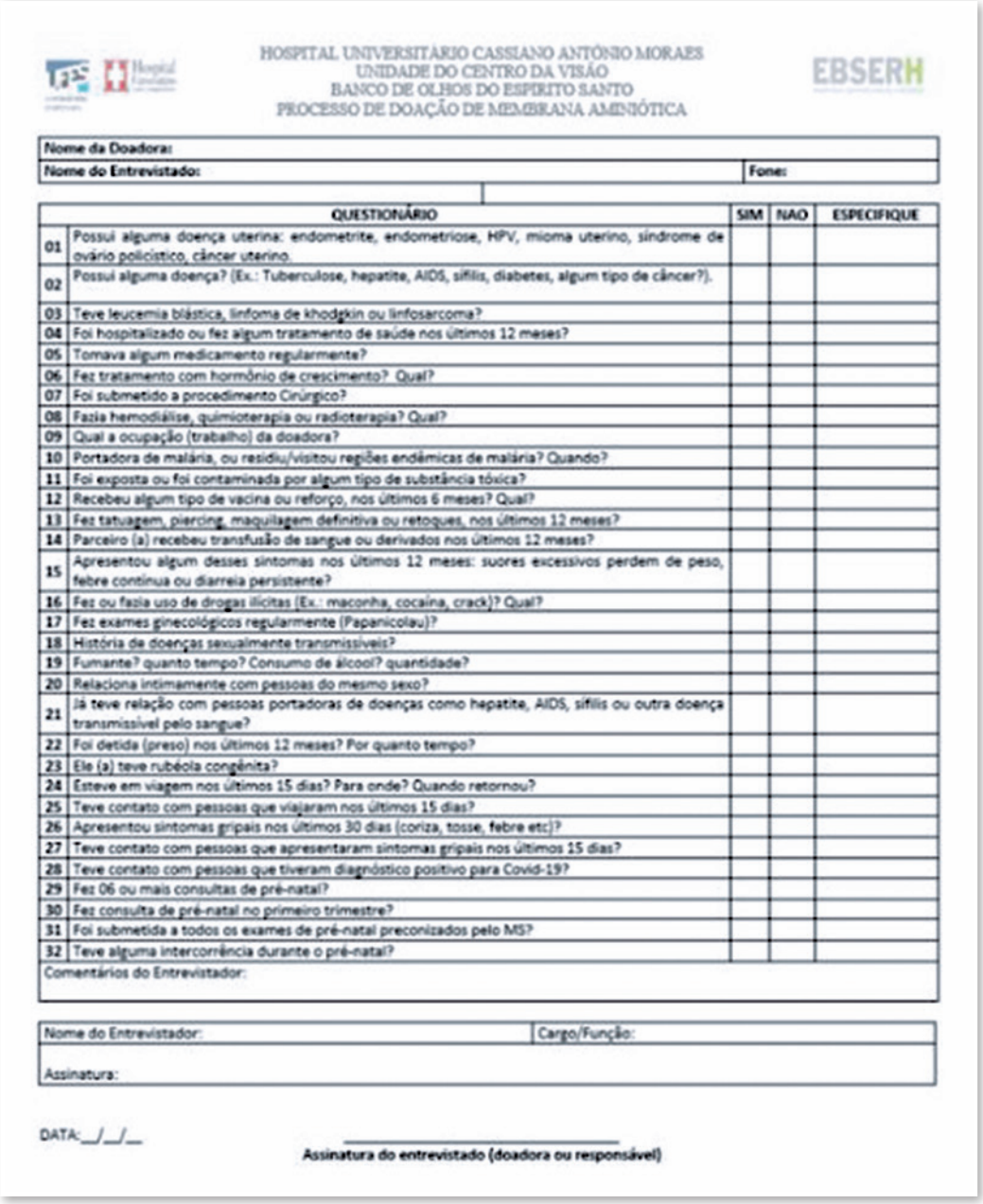
( ) NÃO REALIZADA: _____

MOTIVO

Nº ID LABORATÓRIO:

Figure 4. Placental tissue procurement form and laboratory screening.

c) No history of multiple partners in the last 9 months (mandatory criterion);
d) Evaluation of prenatal screening exams (pregnant woman's card). A positive result in any of the following tests is a criterion for exclusion from this protocol (mandatory criterion): HBsAg, anti-HBc (IgM and IgG), anti-HCV, anti-HIV-1, anti-HIV-2, VDRL, HTLV-1 and -2, CMV (IgM and IgG), toxoplasmosis (IgM and IgG);
e) Preferentially, delivery at term with no confirmed or suspected associated comorbidities such as placental abruption, intrauterine growth restriction, malformations, venereal or congenital infections, meconium during childbirth, acute or chronic fe-



HOSPITAL UNIVERSITÁRIO CASSIANO ANTÔNIO MORAES
UNIDADE DO CENTRO DA VISÃO
BANCO DE OLHOS DO ESPÍRITO SANTO
PROCESSO DE DOAÇÃO DE MEMBRANA AMNIÓTICA

EBSERH

Nome da Doadora:	
Nome do Entrevistado:	**Fone:**

	QUESTIONÁRIO	SIM	NAO	ESPECIFIQUE
01	Possui alguma doença uterina: endometrite, endometriose, HPV, mioma uterino, síndrome de ovário policístico, câncer uterino.			
02	Possui alguma doença? (Ex.: Tuberculose, hepatite, AIDS, sífilis, diabetes, algum tipo de câncer?).			
03	Teve leucemia blástica, linfoma de khodgkin ou linfosarcoma?			
04	Foi hospitalizado ou fez algum tratamento de saúde nos últimos 12 meses?			
05	Tomava algum medicamento regularmente?			
06	Fez tratamento com hormônio de crescimento? Qual?			
07	Foi submetido a procedimento Cirúrgico?			
08	Fazia hemodiálise, quimioterapia ou radioterapia? Qual?			
09	Qual a ocupação (trabalho) da doadora?			
10	Portadora de malária, ou residiu/visitou regiões endêmicas de malária? Quando?			
11	Foi exposta ou foi contaminada por algum tipo de substância tóxica?			
12	Recebeu algum tipo de vacina ou reforço, nos últimos 6 meses? Qual?			
13	Fez tatuagem, piercing, maquilagem definitiva ou retoques, nos últimos 12 meses?			
14	Parceiro (a) recebeu transfusão de sangue ou derivados nos últimos 12 meses?			
15	Apresentou algum desses sintomas nos últimos 12 meses: suores excessivos perdem de peso, febre contínua ou diarreia persistente?			
16	Fez ou fazia uso de drogas ilícitas (Ex.: maconha, cocaína, crack)? Qual?			
17	Fez exames ginecológicos regularmente (Papanicolau)?			
18	História de doenças sexualmente transmissíveis?			
19	Fumante? quanto tempo? Consumo de álcool? quantidade?			
20	Relaciona intimamente com pessoas do mesmo sexo?			
21	Já teve relação com pessoas portadoras de doenças como hepatite, AIDS, sífilis ou outra doença transmissível pelo sangue?			
22	Foi detida (preso) nos últimos 12 meses? Por quanto tempo?			
23	Ele (a) teve rubéola congênita?			
24	Esteve em viagem nos últimos 15 dias? Para onde? Quando retornou?			
25	Teve contato com pessoas que viajaram nos últimos 15 dias?			
26	Apresentou sintomas gripais nos últimos 30 dias (coriza, tosse, febre etc)?			
27	Teve contato com pessoas que apresentaram sintomas gripais nos últimos 15 dias?			
28	Teve contato com pessoas que tiveram diagnóstico positivo para Covid-19?			
29	Fez 06 ou mais consultas de pré-natal?			
30	Fez consulta de pré-natal no primeiro trimestre?			
31	Foi submetida a todos os exames de pré-natal preconizados pelo MS?			
32	Teve alguma intercorrência durante o pré-natal?			
Comentários do Entrevistador:				

Nome do Entrevistador:	Cargo/Função:
Assinatura:	

DATA:__/__/__

Assinatura do entrevistado (doadora ou responsável)

Figure 5. Donor's clinical and social history form.

tal distress, premature rupture of membranes, or endometritis (optional criterion).

Collect blood samples from the donor and fill out the laboratory screening form (Figure 4). Positivity for one (or more) of the tests will make it impossible to use the donated tissue, and it should be discarded in an appropriate place.

2. Collection and storage of placenta and membranes by the gynecology and obstetrics clinic

- Hand hygiene according to the standard operating procedure of the Hospital Infection Control Department;
- After placental expulsion, while properly fitted with personal protective equipment (goggles,

mask, and sterile glove), place the potential donor's placenta and membranes in a collection bag;
- Close and identify the collection bag with the parturient's full name and medical record number;
- Store the collection bag for up to 24 h (from the time of delivery) in an environment with controlled temperature between 2°C and 8°C;
- Discard in an appropriate place any collection bag that has been stored for >24 h.

3. Preparation of the amniotic membrane by the eye bank team of the ophthalmology clinic

3.1. After identifying a donor, completing all documentation, and obtaining authorization for using AM, transport the properly labeled collection bag containing the placenta and its membranes directly to the eye bank in an isothermal box at an internal temperature of 2°C to 8°C.

3.2. Separation and initial preparation of the amniotic membrane

- Hand hygiene according to the institution's protocol;
- Dress with personal protective equipment (goggles, cap, mask, sterile gown, and sterile glove) and distribute the sterile drapes in the work area;
- Separate AM and chorionic membrane from the rest of the placenta using sterile scissors, keeping the epithelial surface of the AM (inner side of the membrane) always facing upwards;
- Return the rest of the placenta to the collection bag;
- Wash the AM and chorion thoroughly with using Ringer's lactate solution;
- Manually separate the chorionic membrane from AM and place the chorion together with the rest of the placenta in a collection bag for proper disposal;
- Arrange two vats and fill them with solution 1 (prepared according to the supplementary file *Description of solutions 1 and 2*);
- AM is divided into two parts, each of which must be placed in a sterile vat or basin and irrigated abundantly with sterile Ringer's lactate for manual and mechanical removal of blood and debris, changing the Ringer's lactate according to need;
- Place the clean membranes in the vats containing solution 1 (Figure 6), and wait 30 min;
- Remove the gloves and sterile gown;
- Sanitize your hands.

3.3. Final preparation of the amniotic membrane samples in a laminar flow hood for storage

- Sanitize hands according to the institution's standard protocol;
- Sanitize the laminar flow hood before handling it according to the institution's standard protocol;
- Arrange the sterile vials for AM storage (Figure 7), according to the mode of preservation to be used in the membranes;

(NOTE: The sterile vial must be selected according to the temperature of the place where it will be preserved. Those intended for temperatures from 2°C to 8°C are made of polypropylene or polyethylene and have a single screw cap, while those made to withstand cold temperatures up to −85°C are made of reinforced polypropylene, with an attached lid, hermetic closure, and double seal.)

- Hand hygiene according to the institution's protocol;
- Dress with personal protective equipment, sterile gown, and sterile glove and distribute the sterile drapes in the work area in the laminar flow hood;
- Introduce the AM storage vials into the laminar flow hood, open them, and place them in the sterile area;
- Arrange the sterile material and the other consumables to be used on the hood bench and open it in such a way as not to contaminate it with the help of an assistant who is not dressed in sterile clothing;
- Arrange nitrocellulose paper, tweezers, scissors, and scalpel on the sterile impermeable drapes;
- Place AM on the nitrocellulose paper with the epithelial surface facing upwards;
- Start cutting the AM in 2.5 × 2.5-cm pieces (Figure 8) and place each piece on nitrocellulose paper in a storage vial containing 10 mL solution 2 (prepared according to the supplementary file *Description of solutions 1 and 2*);
- Place a piece of AM in a tube containing brain–heart infusion (BHI) and send it to the microbiology department (for investigation of fungi, bacteria, nonspecific germs, bacterioscopy, and antibiotic susceptibility testing), incubating for up to 15 days;
- Send a vial containing an AM sample along with the collection bag containing the placenta and chorionic membrane for analysis in the pathology department;
- Discard the blades in the specific box for sharp objects;

Figure 6. Amniotic membrane being placed in vat with solution 1, with the epithelium facing upwards.

Figure 8. Apply the membrane to a nitrocellulose paper and cut into sizes close to 1 inch to be placed in solution 2 for storage.

Figure 7. Storage vial for higher temperatures (A) and cryopreservation storage vial (B).
The vials that can be used for storage of amniotic membrane. Vial (A) is intended for storage at temperatures of 2°C to 8°C, which allows the material to be preserved for up to 3 months. Conversely, vial (B) is indicated for cryopreservation and withstands temperatures from −85°C to −75°C, allowing the material to be preserved for up to 2 years.

- Remove the materials from the hood after the end of the procedure and send them for disposal;
- Remove the personal protective equipment;
- Sanitize hands according to the institution's standard protocol;
- Put on procedure gloves;
- Label the AM storage vials with the identifications of the product (amniotic membrane and size), number of the donation record in the eye bank, name of the person responsible for the preparation, storage temperature, date of preparation, expiration date of the amniotic membrane (2°C to 8°C: 3 months; −85°C to −75°C: 2 years,[10,11]) and seal the vials;
- Keep the jars in the refrigerator (2°C to 8°C) or freezer (-80°C).

4. Monitoring the quality of the amniotic membrane samples by the eye bank team of the ophthalmology clinic

Following the preparation and storage of the AM samples, monthly monitoring of the batches is required through external visual inspection and by sending a sample for microbiological and histopathological analyses. Thus, the material will only continue to be released for use if it does not present alterations on external visual inspection, signs of infection on microbiological and histopathological examination, or signs of advanced tissue necrosis on histopathological examination. The results of these analyses must be stored in the donation record at the eye bank by filling out the sample quality monitoring form (Figure 9).

If any problem is detected with a membrane supplied for use, the physician who used it must notify the eye bank in writing about the problem, and the supply of samples from that batch will be suspended until the situation is investigated with the technician responsible for the eye bank, who will decide whether the samples will be discarded or kept for use. The data must be annotated in the eye bank's donation record.

DISCUSSION

The reality of ophthalmological services in Brazil with regard to the use of AM is still limited to large research centers and universities. Part of this scenario is because of the hospital and laboratory structure necessary to enable the procurement, processing, storage, monitoring, and use of the material in patients with this indication. In view of this, it is extremely important that resolutions such as the one promulgated by the Brazilian National Health Surveillance Agency, Collegiate Board Resolution No. 55 of December 11, 2015[8], be formulated with the aim of standardizing tissue banks. Thus, minimum technical and sanitary requirements should be established for the operation of these facilities, aiming at the safety and quality of the tissues supplied for therapeutic use[8], to enable new application centers to appear and more people to take advantage of this resource.

AVALIAÇÃO DE TECIDO – MEMBRANA AMNIÓTICA
Formulário

DE CÓDIGO BO/ES/MA: _____ ATÉ CÓDIGO BO/ES/MA: _____

TOTAL DE FRAGMENTOS: _____ VALIDADE LOTE: ___/___/_____

DATA DA PRESERVAÇÃO: ___/___/___ HORA: ___:___ RESPONSÁVEL: _____

DATA DA AVALIAÇÃO: ___/___/___ HORA: ___:___ RESPONSÁVEL: _____

INTERVALO DE TEMPO DENTRE A CAPTAÇÃO E A PRESERVAÇÃO: _____

MEIO: GLICERINA PURA FABRICANTE: _____ LOTE: _____
VENC. DO MEIO: ___/___/_____

CLASSIFICAÇÃO DO LOTE

( ) VIÁVEL ( ) INVIÁVEL

OBS: _____

RESULTADO DE EXAMES

___/___/___ DATA ___:___ HORA

_____ RESPONSÁVEL TÉCNICO PROJETO

Figure 9. Tissue quality assessment sheet.

It is obvious that a process with so many steps needs a cohesive multidisciplinary team comprising trained and qualified professionals who will work from the moment of clinical and laboratory screening of the pregnant woman to the monitoring of the sample to enable the use of the material[4]. In addition to the human resources in this process, it is equally essential for users to have access to an adequate structure for the correct storage of the material, laboratory for histopathological analysis, and laboratory for the detection of possible microorganisms contaminating the membrane.

This way it should be possible to produce a surgical graft with good structural quality and little risk of contamination to be used for the benefit of patients with ophthalmic diseases. In addition, the preservation and long-term storage of biological materials not only offers the advantage of good availability for a longer period but it also reduces the risk of disease transmission[10,11].

The use of AM in ophthalmology is one of the main therapeutic options for ocular surface diseases refractory to clinical treatment, especially those involving alterations in the healing process and the ocular physical barrier. To date, no articles have been published in the scientific literature to guide the collection, preparation, storage, and monitoring of human AM samples according to Brazilian and international quality standards. The development of a clinical protocol as described in this article is essential to ensure the quality of AM samples, reduce risks to patients, and popularize the use of this tissue in daily ophthalmological practice.

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Description of solutions 1 and 2

1. Solution 1

1.1. Items required (all with sterile contents)

Substance and presentation	Quantity and dilution
Gentamicin - Ampoule, 80mg/2mL	Dilute 1 ampoule from 2mL to 10 mL using distilled water
Benzathine penicillin - Ampoule, 1,200,000U/4mL	Dilute 1 ampoule to 20mL using distilled water
Ciprofloxacin - Pouch, 200mg/100mL	1 pouch, protected from light
Amphotericin B - Ampoule 50mg	Dilute 2 ampoules to 20mL with distilled water, protected from light
Distilled water - Ampoule, 10mL	5 ampoules
Ringer lactate solution - Pouch, 1000mL	One 1-L pouch (850mL will be used)

1.2. Final concentrations

Gentamicin 80μg/mL
Benzathine penicillin 1200U/mL
Ciprofloxacin 200μg/mL
Amphotericin B 100μg/mL

1.3. Final volume

– 1 L

1.4. Preparation

Mix 10mL gentamicin solution, 20mL benzathine penicillin solution, one 100-mL pouch of ciprofloxacin, and 20mL amphotericin B solution with 850mL Ringer's lactate solution (to complete a final volume of 1L solution).

2. Solution 2

2.1. Items required (all with sterile contents)

Substance and presentation	Quantity and dilution
Gentamicin - Ampoule, 80mg/2mL	Dilute 1 ampoule from 2mL to 10mL-5mL will be used
Benzathine penicillin - Ampoule, 1,200,000U/4mL	Dilute 1 ampoule to 20mL using distilled water-10mL will be used
Ciprofloxacin - Pouch 200mg/100mL	1× 100-mL pouch, protected from light-50mL will be used
Amphotericin B - Ampoule 50 mg	Dilute 2 ampoules to 20mL with distilled water, protected from light
Distilled water - Ampoule 10mL	8 ampoules
Glycerin P.A. - 1000mL bottle	2× 1-L bottles (925mL will be used from each bottle)

2.2. Final concentrations

Gentamicin 40μg/mL
Benzathine penicillin 600U/mL
Ciprofloxacin 100μg/mL
Amphotericin B 50μg/mL

2.3. Final volume

– 1 L

2.4. Preparation

– Mix well 5mL gentamicin solution, 10 mL benzathine penicillin solution, 50mL ciprofloxacin, and 10 mL amphotericin B solution with 925mL glycerin P.A. (to complete a final volume of 1 L solution). Repeat the preparation to produce 2 L solution 2.

3. Technical Notes

3.1. Solutions 1 and 2 were based on antimicrobials already applied in other tissue storage processes[4], and changes were made in the choice of medications considering their spectrum of action and availability.

3.2. Antimicrobials and their respective concentrations used in the searched references: 50μg/mL penicillin, 50μg/mL streptomycin, 100 μg/mL neomycin, and 2.5μg/mL amphotericin B. (4)

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