Microbiological viability of bovine amniotic membrane stored in glycerin 99% at room temperature for 48 months

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10.1590/0034-737X201764010008

ABSTRACT

The medium for storing biological tissues is of great importance for their optimal use in surgery. Glycerin has been proven efficient for storing diverse tissues for prolonged time, but the preservation of the bovine amniotic membrane in glycerin 99% at room temperature has never been evaluated to be used safely in surgical procedures. This study evaluated the preservation of 80 bovine amniotic membrane samples stored in glycerin 99% at room temperature. The samples were randomly divided evenly into four groups. Samples were microbiologically tested after 1, 6, 12 and 48 months of storage. The presence of bacteria and fungi in the samples was evaluated by inoculation on blood agar and incubation at 37 ºC for 48 hours and on Sabouraud agar at 25 ºC for 5 to 10 days. No fungal or bacterial growth was detected in any of the samples. It was concluded that glycerin is an efficient medium, regarding microbiology, for preserving pre-prepared bovine amniotic membrane, keeping the tissue free of microorganisms that grow in the media up to 48 months at room temperature.

Key words: biological membranes; preservation medium; surgery; amnion; caesarean section.

RESUMO

Viabilidade microbiológica de membrana amniótica bovina armazenada por 48 meses em glicerina 99% em temperatura ambiente

O meio de armazenamento dos tecidos biológicos tem grande importância para que os mesmos possam ser empregados da melhor forma em cirurgias. A glicerina tem se mostrado eficiente, considerando aspectos microbiológicos, em armazenar alguns tecidos por períodos prolongados. Porém, nunca foi avaliada a conservação da membrana amniótica bovina em glicerina 99% em temperatura ambiente para que, posteriormente, pudesse ser empregada com segurança em procedimentos cirúrgicos. Assim, foram avaliadas 80 amostras de membrana amniótica bovina armazenadas em glicerina 99% em temperatura ambiente e divididas aleatoriamente em quatro grupos de igual número. As amostras foram submetidas à avaliação microbiológica após 1, 6, 12 e 48 meses de armazenamento. Com o objetivo de avalizar a possível presença de bactérias e fungos nas amostras, elas foram inoculadas em ágar-sangue e incubadas a 37 ºC por 48 horas e em ágar Sabouraud a 25 ºC durante 5 a 10 dias. Não houve crescimento fúngico ou bacteriano em nenhuma das amostras. Conclui-se que a glicerina é um meio de conservação eficiente, no que tange a microbiologia, de membrana amniótica bovina previamente preparada, mantendo o tecido isento de microorganismos que se multiplicam nos meios utilizados por um período de até 48 meses em temperatura ambiente.

Palavras-chave: membranas biológicas; meios de conservação; cirurgia; âmnion; operação cesariana.

Submitted on June 12th, 2015 and accepted on December 19th, 2016.

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INTRODUCTION

Sterility maintenance is a primary concern regardless of the method for preserving a biomaterial (Slatter & Dietrich, 2007). Thus, a number of media have been studied for storing biological membranes and glycerin has shown good results (Okamoto *et al*., 2000). Glycerin has antiseptic properties, acts as fixative and dehydrating agent (Chirife *et al*., 1982; Amendola, 2007), is capable of preserving the ionic concentration and cellular integrity of the tissue (Pigossi, 1967), and is indicated for the preservation of numerous biological materials. Its low cost and easy handling also encourage its use as storage medium (Pigossi, 1967; Amendola, 2007; Pontes *et al*., 2008; Pontes *et al*., 2011).

On the basis of the authors' clinical and experimental experience, the amniotic membrane has already been used in the treatment of superficial and penetrating experimental corneal ulcers in rabbits (Pontes *et al*., 2008; Pontes *et al*., 2014), corneal ulcers refractory to clinical treatment in dogs, and after resection of feline corneal sequestrum (Pontes *et al*., 2010) and dermoid cyst in dogs (Rios *et al*., 2014). In veterinary medicine, it has already been used in the treatment of penetrating corneal ulcers (Barros *et al*., 1998), resection of corneal and scleral tumors, in symblepharon treatment (Barros *et al*., 2005), limbic cell deficiency (Cremonini *et al*., 2007), and chemical burns (Choi *et al*., 2011). This type of membrane can be used fresh or preserved in some type of medium. The amniotic membrane is harvested during cesarean section of term fetuses, making it impracticable to apply it fresh in surgeries without previous planning. In this context, when the amniotic membrane is necessary in a certain surgical procedure, it should be available in a tissue bank and stored in a medium that prevents bacterial and fungal growth. It is, therefore, important the creation of amniotic membrane banks. Besides, it is not difficult to acquire amniotic membranes, especially in the veterinary context, which makes it an attractive biomaterial for use in surgeries.

Currently, the use of amniotic membrane has become common in surgeries, but there are no reports in the literature on the suitability of glycerin 99% at room temperature as a microbiological conservation medium for this type of biomaterial.

Thus, the objective of this study was to evaluate the use of glycerin 99% for preserving bovine amniotic membrane at room temperature for 48 months. If its efficiency was demonstrated during this period, banks of this biomaterial could be created, allowing its use over time in several types of surgical procedures, without having to plan a caesarean section for harvesting the amniotic membrane at the time coinciding with its use in fresh.

MATERIAL AND METHODS

This research project was approved by the Research Ethics Committee of the Faculdade de Ciências Biológicas e da Saúde - FACISA / UNIVIÇOSA, number 00113/2012.

The amniotic membrane was prepared according to the literature (Kim & Tseng, 1995); it was harvested under sterile conditions from a pregnant female bearing a full-term fetus during elective caesarean section at the Veterinary Hospital of the Universidade Federal de Viçosa. In a surgical environment, the amniotic membrane, with the chorion, was first washed in 0.9% sterile saline solution at room temperature to remove the clots, then washed three times with sterile phosphate buffer solution containing 1000 IU/ml penicillin G, 20 mcg/ml streptomycin, and 2.5 mcg/ml amphotericin B. The amnion was manually separated from the chorion (Figure 1A) (Kim & Tseng, 1995), mounted, epithelial face upwards, onto nitrocellulose paper (Figure 1B), and cut into the size of 1.0 cm^2. Subsequently, the fragment was placed in a sterile glass vial and covered with sterile glycerin 99% (Figure 1C) (Pontes *et al*., 2014).

A total of 80 flasks containing amniotic membrane in glycerin 99% were randomly distributed into four equally sized groups. Each group contained 20 bovine amniotic membrane fragments and were all maintained at room temperature. Thirty days after harvest, group 1 was microbiologically tested (Barros *et al*., 1998, Pontes *et al*., 2008, Pontes *et al*., 2014). Group 2 was subjected to the same test after 6 months, group 3 after 12 months, and group 4 after 48 months.

Membrane fragments were aseptically collected using sterile tweezers in a laminar flow cabinet, triturated with 0.85% sterile saline solution and inoculated by surface spreading on plates containing blood agar (5% sheep's blood) and plates containing Sabouraud dextrose agar. To isolate bacteria, the samples inoculated on blood agar and incubated at 37 ºC for 48 hours and to monitor fungal growth, the samples were inoculated on Sabouraud agar at 25 ºC for 5 to 10 days (Quinn *et al*., 2005).

RESULTS AND DISCUSSION

Fungal growth was observed in two samples of G4. Therefore, a new microbiological analysis was performed in all the samples and none of them showed fungal or bacterial growth. This result suggests that there was contamination during the handling of the membranes in the first microbiological analysis, corroborating with Gioso *et al.* (2002), who found that the simple handling of bones, however careful, could introduce contaminating microorganisms into the samples.

The amniotic membrane was microbiologically evaluated only after 30 days, because according to literature, the membrane should remain for at least 30 days

in glycerin for the loss of the ability to stimulate immune reaction to take place (Pigossi, 1967; Daleck *et al*. al., 1992; Costa Neto *et al*., 1999).

Additionally, elimination of bacteria may require up to 27 days to occur and the tissue should remain for at least 30 days in glycerin, according to a study on the effects of glycerin on tendons inoculated with gram negative and gram positive bacteria (Krauspenhar *et al*., 2003).

Although the amniotic membrane has been properly prepared according to the literature (Kim & Tseng, 1995), we can attribute to glycerin 99% the absence of fungal and bacterial growth during storage of bovine amniotic membrane at room temperature for up to 48 months. This result was important, since glycerin had never been microbiologically evaluated as a medium for preserving the amniotic membrane at room temperature. Glycerin has been studied for storing pericardium (Brun *et al*., 2002a) and bone tissue (Gioso *et al*., 2002), which in the long term showed no significant growth of microorganisms.

Amniotic membrane preservation has been reported in glycerin at 4 °C (Kim & Tseng, 1995) and at -80 °C (Dekaris & Gabriæ, 2009), but our objective was to evaluate the microbiological efficiency of glycerin 99% in preserving the tissue at room temperature. A number of authors found that glycerin in association with a cell culture medium MEM (Minimal Essential Medium), at -80 ºC, acted to guarantee the tissue integrity and the preservation of growth factors and cytokines in human amniotic membrane (Pena *et al*., 2007), but others concluded that glycerin used as a cryoprotectant may impair the function of human amniotic membrane as a substrate for limbus cell culture (Shortt *et al*., 2008). Thus, the other effects that glycerin 99% used as storage medium can cause in the amniotic membrane is still controversial.

Because our group frequently uses the amniotic membrane in research and in the hospital routine (Pontes *et al*., 2011; Pontes *et al*., 2014), it was important to evaluate this medium aiming at creating a bank of this tissue to be used in the long term.

Some other factors were also taken into account to evaluate glycerin 99% as a preservation medium, including low cost and easy handling (Pigossi, 1967; Amendola, 2007; Pontes *et al*., 2011; Pontes *et al*., 2014) and acquisition (Pontes *et al*., 2008), as well as abolishing other high-cost media of difficult transportation (Alvarenga, 1992) such as keeping at -80 °C.

The ability of glycerin 99% to act against some specific bacterium and fungus was not evaluated, since it was not the objective of this study. However, some studies have confirmed its bactericidal action on *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginos* and its inability to prevent the growth of *Clostridium botulinium* and *Clostridium perfringes* (Pigossi *et al*., 1971; Amendola, 2007). Other studies reported that glycerin has no action on some more resistant sporulated forms (Pigossi *et al*., 1971) or viruses (Coronado *et al*., 1998), while some authors reported that glycerin is only effective against type I herpes virus and poliovirus when at 98% (Marshall *et al*., 1995). The same authors stated that to be effective against these viruses, the tissue must be maintained in 98% glycerin for at least 4 weeks at up to 20 °C.

As sterility maintenance is a primary concern in any conservation method (Slatter & Dietrich, 2007), several media have already been analyzed for the preservation of biological membranes, including glutaraldehyde (Rabelo *et al*., 2004; Khor, 1997; Oliveira *et al*., 2009), 300% glucose solutions (Oliveira *et al*., 2009; Brun *et al*., 2002b; Melo Filho *et al*., 2011; Vidor, 2012), and 5% polyvinylpyrrolidone (Mota *et al*., 2002), which presented advantages and disadvantages.

By analyzing the various media and methods of preservation for biological material in the literature, it can be suggested that glycerin 99%, at room

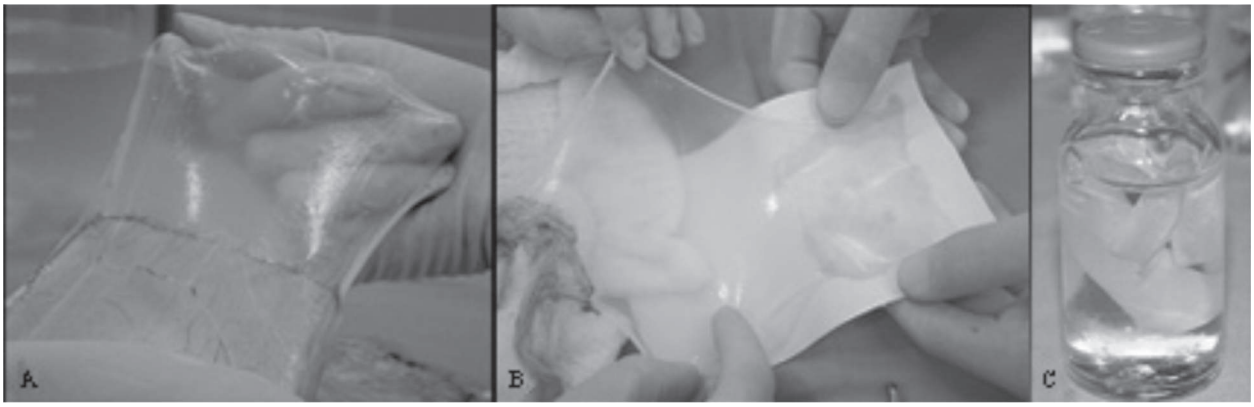

Figure 1: Pictures showing bovine amniotic membrane preparation. A - Amnion being manually separated from the chorion. B - Amniotic membrane spreading onto nitrocellulose paper with epithelial face upwards. C - Amniotic membrane fragments in a flask containing sterile glycerin 99% to be stored at room temperature.

temperature, shows greater advantages when used to store the bovine amniotic membrane under the described conditions. This finding makes it an ideal medium to be used in the creation of a bank of this type of membrane, with the possibility of its microbiological conservation at room temperature for long periods. This fact may facilitate the use of bovine amniotic membrane in the hospital routine, since it is a biomaterial of easy acquisition and handling, reducing discarding because of the impossibility of using it in fresh.

CONCLUSIONS

Glycerin 99% is an efficient preservation medium for bovine amniotic membrane previously prepared as described, with respect to microbiology, keeping the tissue free of microorganism growth in the media kept at room temperature for up to 48 months.

ACKNOWLEGEMENTS

The authors want to thank the Núcleo de Pesquisa e Extensão (NUPEX) of the Faculdade de Ciências Biológicas e da Saúde (FACISA / UNIVIÇOSA), the Universidade Federal de Viçosa (UFV), Ophthalmos Indústria Farmacêutica, the Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológio (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the support granted.

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