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ORIGINAL ARTICLE

Microbiological Evaluation of Different Preservative Methods for Amnion Graft

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ABSTRACT

Background: *The use of fetal membranes as a graft has gained a lot of attention since 1910 and ever since it has been used in clinical practice notably in ocular surgeries and in the treatment of burns and skin ulcerations. Amnion graft should be sterile, free of contamination, easily obtained and can be stored for long periods of time without affecting graft integrity. Methods currently used to preserve amnion graft include hypothermic (fresh) storage, freezing, freeze drying and gamma irradiation. For any storage method selected assessing any potential bacterial or fungal contamination is profoundly important to ensure the safety of the stored graft.* ***Objectives:*** *In this study, five different preservative media commonly used in Egypt for storing amnion graft hypothermally were evaluated microbiologically to select the optimum preservation method.* ***Methodology:*** *A total of 40 amnion grafts were obtained from consented women delivered by elective caesarean section. The graft of each donor was split into 7 pieces, two pieces from each donor were cultured immediately after collection to exclude contaminated grafts. The remaining 5 pieces of non-contaminated grafts were stored in five preservative media at 4°C for three weeks: Sodium hypochlorite 0.025% , Glycerol 85%, Gentamycin (0.32 mg/ml) in 100% Phosphate-buffered saline (PBS), Penicillin G (50,000 IU/ 100 ml), and a mixture solution comprised of Streptomycin (50mg/ml), Penicillin (50 mg/ml), and Amphotericin B (2.5 mg/ml) were used for preservation. By the end of each week the pieces were cultured onto aerobic and anaerobic media and the recovered colonies were identified.* ***Results:*** *25 grafts out of 40 were included in the study. The findings of the study revealed that the contamination was observed in three preservative media, the highest of which was Penicillin G followed by Garamycin and the least contamination proportion was identified in the mixture solution (96% 88% and 44% respectively). The contaminants were mainly Filamentous fungi (69.3%) and Candida albicans (4%) while Staphylococcus aureus was only recovered in two specimens (2.6%). Glycerol 85% and Sodium hypochlorite were free from contamination all over the storage period.* ***Conclusion:*** *The preservation of amnion graft in glycerol 85% and sodium hypochlorite 0.025% were proved to be the optimum media for preservation up to three weeks from a microbiological perspective.*

INTRODUCTION

Human amnion graft is derived from the fetal membrane which consist of a single layer of amnion cells fixed to collagen-rich mesenchyme and loosely attached to the chorion. The use of fetal membranes as a graft has gained a lot of attention since 1910 and ever since it has been used in clinical practice notably in ocular surgeries and in the treatment of burns and skin ulcerations[1]. Amnion graft is rich in biologically active factors which promotes healing and serves as an effective material for dressing of wounds. Further, it enhances epithelialization with anti-fibrotic, anti-inflammatory, anti-angiogenic and anti-microbial features[2].

It is better to be collected from a consenting donor by Caesarean Section (CS) to avoid structural defects associated with stretching of the graft during normal vaginal delivery and/or any potential contamination by normal vaginal flora[3]. However, storing the graft in a contaminant free milieu and at an optimum storage temperature have been always a challenge. Methods used to preserve amnion graft include fresh (hypothermic) storage, freezing, freeze drying and gamma irradiation. The optimum preservative method would be the one that keeps the integrity of the graft and helps promoting rapid re-epithelisation[4].

Given that banked frozen amnion graft is unavailable or inaccessible in some settings in Egypt, hypothermic storage provides a feasible and convenient substitute when the used preservative can effectively disinfect the stored tissue[5].

Glycerol, sodium hypochlorite and various mixtures of antibiotics and antifungals with different concentrations have been investigated to advise on the method that can best keep the integrity of graft and preserve it as long as it is needed without the risk of transmission of infection.

Additional virologic testing is to be conducted further at the time of donation and 6 month later, and upon receipt of negative blood test results the stored tissue will be released for grafting[6,7].

In this study, five different preservative media commonly used in Egypt were assessed for any potential bacterial or fungal contamination to ensure the safety of transferred graft.

METHODOLOGY

A total of 40 amnion grafts were obtained from consenting women delivered by elective CS at the Department of Obstetrics and Gynecology, Ain Shams University Hospital. The selection criteria included: 1) pregnant women without a history of premature rupture of membranes (P.R.O.M.), endometritis, Jaundice, toxemia or malaria 2) seronegative for hepatitis B, C, HIV, and Syphilis 3) grafts that are not stained with meconium. Approval of the ethics committee at Faculty of Medicine, Ain Shams University was taken for the research design.

The placenta was taken immediately following the elective CS under strict aseptic conditions. The amnion graft was separated easily from the remaining chorion by blunt dissection and rinsed several times in saline to remove excessive blood clots. The graft of each donor was split into 7 pieces under complete aseptic condition: two pieces from each donor were cultured onto in aerobic and anaerobic media immediately after collection to exclude contaminated grafts. The remaining 5 pieces were preserved in five preservative media and stored at 4°C in the Medical Microbiology and Immunology Laboratory for three weeks. By the end of each week the pieces were cultured onto aerobic and anaerobic media and the recovered colonies were identified.

The media used for amnion grafts preservation were ready made solutions purchased from the local market and comprised of: Sodium hypochlorite 0.025%, Glycerol 85%, Garamycin (0.32 mg/ml) in 100% Phosphate-buffered saline (PBS), Penicillin G (50,000 IU/ 100 ml), and a mixture solution comprised of Streptomycin (50mg/ml), Penicillin (50 mg/ml), and Amphotericin B (2.5 mg/ml), (hereinafter referred to as the mixture).

Bacteriological examination:

Two pieces of amnion graft from each donor were cultured both aerobically and anaerobically immediately after the collection to exclude any bacterial or fungal contamination during the sampling procedure. Any contaminated specimen was discarded, so that any bacterial or fungal isolation will be a direct result of the preservative procedure rather than sampling aseptic techniques errors. Amnion grafts from the different preservative media were cultured one week, two weeks and three weeks after sampling procedure (Figure 1)

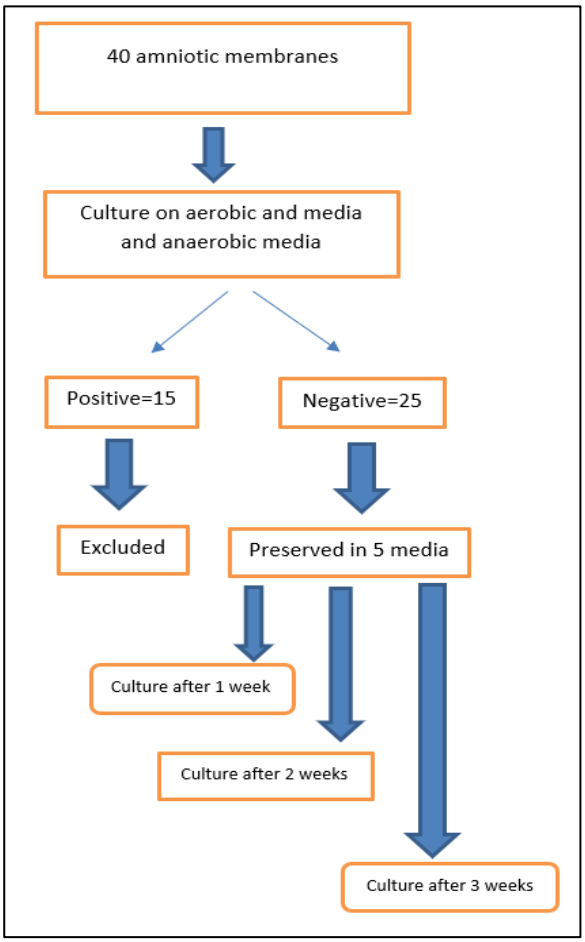

Fig. 1: Algorithm demonstrating the steps of amnion graft processing

The media used for isolation were Blood agar, MacConkey and Sabaroud's dextrose agar. The recovered contaminants were identified according to the conventional bacteriological methods by *Colle et al.*,[8].

Statistical Analysis

Data are expressed as mean ±SD (range) or as number (%) of cases. The results were statistically analyzed using the McNemar test, with a statistical significance index of 0.05.

RESULTS

Out of 40 amnion grafts selected from women delivered by CS according to the stated criteria, 15 grafts were excluded due to bacterial contamination and 25 grafts were free from any bacterial or fungal contamination and therefore were included in the study.

Culture results over three weeks period were recorded by the end of each week. Culture results on the third week of preservation showed that the contamination was observed in three preservative media, the highest of which was Penicillin G (96%) followed by Garamycin (88%) and the least contaminated preservative method was the mixture (44%) (Table 1).

Table 1: Proportion of bacterial and fungal contamination in various preservative media

The type of the preservant	Contamination	Immediately after collection (n=25)		After 1 week (n=25)		After 2 weeks (n=25)		After 3 weeks (n=25)	
		n	%	n	%	n	%	N	%
Sodium hypochlorite 0.025%	-ve	25	100%	25	100%	25	100%	25	100%
	+ve	0	-	0	-	0	-	0	-
Glycerol 85%	-ve	25	100%	25	100%	25	100%	25	100%
	+ve	0	-	0	-	0	-	0	-
Garamycin 0.32 mg/ml in 100% PBS	-ve	25	100%	11	44.0	6	24.0	4	16.0
	+ve	0	-	14	**56.0**	19	**76.0**	22	**88.0**
Penicillin G 50.000 IU /ml	-ve	25	100%	7	28.0	3	12.0	2	8.0
	+ve	0	-	18	**72.0**	22	**88.0**	24	**96.0**
Mixture of Penicillin, Streptomycin and Amphotericin B	-ve	25	100%	18	72.0	14	56.0	13	52.0
	+ve	0	-	7	**28.0**	11	**44.0**	11	**44.0**

The recovered colonies were identified according to conventional methods. The identified colonies were mainly *C.albicans*, *S.aures* and *Filamentous fungi*. *C.albicans* was identified using Gram stain and confirmed with Germ tube test. *S.aureus* was identified using Gram stain and confirmed with coagulase test. Fungi other than *C.albicans* were identified at Mycology Center, Assiut University, Faculty of Science as *Rhizopus rhizopodiformis* and *Aspergillus flavus* (hereinafter referred to as *Filamentous fungi*), indicating that the source of contamination is most likely to be environmental.

Culture results on third week revealed that *Filamentous fungi* were detected in the three contaminated preservatives: Penicillin G, Garamycin and the mixture (88%, 76% and 44% respectively). *C.albicans* was detected in two; Penicillin G and Garamycin (8% and 4% respectively), while *S.aureus* was only detected in grafts preserved in Garamycin (8%) (Figure 2).

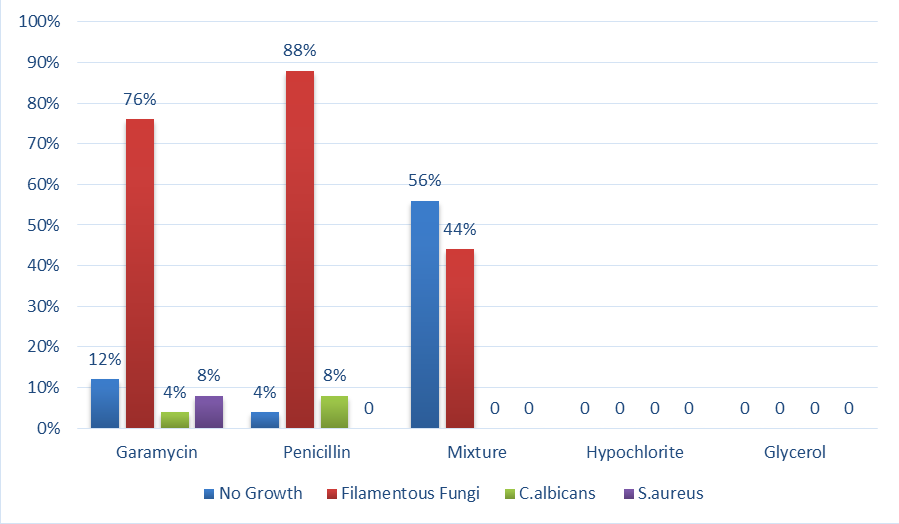

Fig. 2: Culture results on the third week of preservation

Amnion grafts preserved in Garamycin were significantly (P<0.001) contaminated with *Filamentous fungi* and this rate increased steadily from first week till the third week of preservation (56%, 72% and 76% respectively). Contamination with *C.albicans* and *S.aureus* remained constant throughout the second and third week (4% and 8% respectively) (Table 2).

Table 2: Culture results of amnion grafts preserved in Garamycin (0.32mg) in 100% PBS

Culture results (n=25)	Immediately after collection		One week after		2 weeks after		3 weeks after	
	n	%	n	%	n	%	n	%
No growth	25	100	10	40	4	16	3	12
Candida albicans	0	0	1	**4**	1	4	1	4
Filamentous fungi	0	0	14	**56**	18	**72**	19	**76**
Staphylococcus aureus	0	0	0	0	2	8	2	8
Total	25	0	25	100	25	100	25	100
P			<0.001		<0.001		<0.001	

P<0.05 = significant

Amnion grafts preserved in Penicillin G were significantly (P<0.001) contaminated with *Filamentous fungi* and this rate increased gradually from first week till the third week of preservation (72%, 84% and 88% respectively). Contamination with *C. albicans* did not vary between second and third week of preservation (8%) (Table 3).

Table 3: Culture results of amnion grafts preserved in Penicillin G

Culture results(n=25)	Immediately after collection		One week after		2 weeks after		3 weeks after	
	n	%	n	%	n	%	n	%
No growth	25	100	7	28	2	8	1	4
Candida albicans	0	0	0	0	2	8	2	8
Filamentous fungi	0	0	18	72	21	84	22	88
Staphylococcus aureus	0	0	0	0	0	0	0	0
Total	25	0	25	100	25	100	25	100
p			<0.001		<0.001		<0.001	

P<0.05 = significant

Amnion grafts preserved in the mixture were significantly (P<0.001) and only contaminated with *Filamentous fungi* from first week till the third week of preservation (24%, 40% and 44% respectively) (Table 4).

Table 4: Culture results of amnion grafts preserved in the mixture of Penicillin, Streptomycin and Amphotericin B

Culture results (n=25)	Immediately after collection		One week after		2 weeks after		3 weeks after	
	n	%	n	%	n	%	n	%
No growth	25	100	19	76	15	60	14	56
Candida albicans	0	0	0	0	0	0	0	0
Filamentous fungi	0	0	6	24	10	40	11	44
Staphylococcus aureus	0	0	0	0	0	0	0	0
Total	25	0	25	100	25	100	25	100
p			0.02		<0.001		<0.001	

P<0.05 = significant

DISCUSSION

In this work, we aimed to evaluate the different preservative media for amnion graft and identify the optimum method based on the level of contamination in addition to the cost and complexity of the method used.

To our knowledge, this is by far the first study that compared five different preservative media for amnion graft.

In the current study, amnion grafts of 25 women delivered by elective Caesarean Section were obtained and stored at 4°C in five different preservative media.

The study confirmed that the hypothermal preservation of amnion grafts

in Sodium hypochlorite 0.025% and Glycerol 85% were safe and free of contamination based on microbiological testing throughout the 3 weeks. Additionally, both preservatives are feasible, simple and available in the local market.

Our findings were also supported by Ganatra[9], who reported that preservation of amnion graft in Sodium hypochlorite 0.025% was safe and reliable for 30 days. Its excellent bactericidal and fungicidal activity makes it the best option for disinfection and decontamination.

Similarly, Glycerol 85% showed no signs of growth on culture media till the third week of preservation. This finding was also supported by Tugrul Maral et al.[10], Ravishanker et al.[11] and Zidan et al.[12]. All recommended the use of Glycerol for preservation of amnion graft due to its strong antiviral and antibacterial activity minimizing the risk of disease transmission.

In contrast to aforementioned results, Penicillin G was only efficient during the first week of preservation. Significant contamination with *C. albicans* and *Filamentous fungi* were observed during the second and third week (8% and 84-88% respectively) which can be explained by the fact that Penicillin inhibits bacterial cell wall synthesis, and therefore cannot inhibit fungal cell wall which completely differs in structure. Further, Candida and other fungal species await any opportunity offered by antibacterial compound to overgrow without any bacterial competition[13]. The combination and concentration of antibiotics was also controversial. A Penicillin- Streptomycin combination has been most suggested in literature. Penicillin and Streptomycin are bactericidal. Conceptually, amnion graft preserved in mixture of Penicillin, Streptomycin and Amphotericin B was only effective during the first week and *Filamentous fungi* infection was significantly observed during second and third week (40%, 44% respectively). Amphotericin B efficiency against *Filamentous fungi* is variable and least effective compared to other antifungal agents. However, it is the antifungal of choice in treating *C. albicans*, hence none was detected in all samples[14].

This study reported that the preservation of the samples of amnion graft in Garamycin 32 mg/ml an antibiotic that inhibit protein synthesis was contaminated with *Filamentous fungi* starting form first week and steadily increasing till the third week of culture (56%, 72%, 78% respectively). In contrast, *C.albicans* and *S.aureus* were identified on the second and third week with a similar proportion (4% and 8% respectively). The antibiotic is a potent bactericidal against Gram negative bacteria with less inhibitory effect against *S.aureus*, and ineffective against *Filamentous fungi* and *C.albicans*[13]. Csiinge et al.[7] reported that the use of Penicillin Streptomycin or other aminoglycoside combinations were ineffective in the preservation of grafts.

The source of contamination could be environmental for *Filamentous fungi*, while for *C. albicans* and *S. aureus* could be attributed to incomplete aseptic procedures during processing, preservation and/or repeated culture of amnion grafts.

In conclusion, amnion graft should be sterile, free of contamination, easily obtained and can be stored for long periods of time without deterioration. Therefore, based on the results of the current study, the preservation of amnion graft in glycerol 85% or in sodium hypochlorite 0.025% proved to be safe up to three weeks. Other factors such as graft integrity and duration of re-epithalization should also be considered.

Conflicts of interest: The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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