Original Article

EVALUATE APOPTOSIS, NECROSIS AND VIABILITY OF HUMAN SPERM AFTER SEMEN PROCESSING AND CRYOPRESERVATION

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Abstract

Background: Sperm viability of human spermatozoa in Assisted Reproductive Technique(ART) is one of the major concerns as many changes occur in sperm cells as a result of Cryopreservation, including membrane abnormalities and cell death namely apoptosis and Necrosis.

Aim: The prime motive of this study is to identify the best washing method for Oligoasthenospermic patients who undergo ART by analyzing early apoptosis, late apoptosis and necrosis after cryopreservation of sperm for a period of three months.

Study Setting and Design: Three methods of sperm washing viz. Density Gradient, Swim-up and Magnetic Activated Cell Sorting methods were used for comparative analysis for which semen samples were obtained from 30 Oligoasthenozoospermia patients with progressive motility of less than 32%, who underwent ART procedure at Sumathi Fertility Centre, Madurai between June-October 2021. Informed consent was obtained from all men prior to the study. This research was approved by the institutional review board of Bharath University, Chennai.SBDCH/IEC/02/2017/09.Materials and Methods: Briefly, the semen sample was subjected to the three washing methods. After washing, the samples were cryopreserved in liquid nitrogen for a period of 3 months. After 3 months Apoptosis, Necrosis and viability were assessed by using EtBr and Acridine orange stain.

Statistical Analysis: IBM SPPS version 2.0 was deployed to measure the viability of semen using the three washing method.

Results: The swim-up method had higher early and late apoptosis seen in it and a Yellowish green colour showed early apoptosis and an Orange colour was seen with respect to late apoptosis. The viable cells were seen more in the Density gradient method whereas the Magnetic Cells sorting method showed increased levels of Necrosis. The density gradient method more viable able cells compared to swim-up and magnetic activated and cell sorting methods. Early and late apoptosis is more seen in the Swim-up method unsuitable for ICSI and IVF. The magnetic method shows high necrosis in semen processing and is unsuitable for the ICSI method.

Keywords: Phosphatidylserine (PS), Apoptosis, Oligospermia, Asthenospermia

Introduction:

Many alterations occur in mammalian spermatozoa after cryopreservation and thawing [1]. Reduced motility and membrane alterations, such as sperm capacitation or acrosomal response, are the most common types of damage caused by cryopreservation. The viability of sperm is affected by cryopreservation.

Apoptosis and necrosis are two different types of cell death. On the other hand, Apoptosis is a biologically regulated cell death that affects single cells without generating inflammation in the surrounding tissue [2, 3]. Necrosis is caused by injury and affects vast numbers of cells, causing cell swelling and membrane rupture. Apoptosis is a three-phase process that can be broken down into induction, execution, and degradation. Mitochondria are known to play a key function in the execution phase of the cell cycle. Mitochondrial holes open after apoptosis is induced, resulting in a drop in mitochondrial membrane potential (m). Proapoptotic substances from the mitochondria are released by the opening of mitochondrial pores [4]. The proapoptotic factors, for example, various caspases family proteases (cysteine proteases with aspartate specificity)—are then activated in the cytoplasmic compartment, leading to the breakdown phase. Both the cell surface and the nucleus undergo alterations during this period. Phosphatidylserine (PS) is normally sequestered in the inner leaflet of the plasma membrane, but it appears in the outer leaflet, where it prompts noninflammatory phagocytic identification of the apoptotic cell [5]. Internucleosomal cleavage of DNA by particular endonucleases creates 180-base pair DNA fragments in apoptotic cells [2]. Apoptosis efficiency is required for normal spermatogenesis.

Gorczyca et al. [6] were the first to notice spermatozoa with DNA fragmentation, which is similar to death in somatic cells. They theorized that this event would render aberrant ejaculated spermatozoa inactive, which might be harmful. If these defective germ cells are not removed efficiently during spermatogenesis, there will be a large number of aberrant sperm cells in the sperm, resulting in reduced fertility. Few researchers have looked at the effects of cryopreservation on sperm cell apoptotic manifestations. Cryopreservation is linked to membrane PS translocation in human [7–8] sperms, according to the researchers.

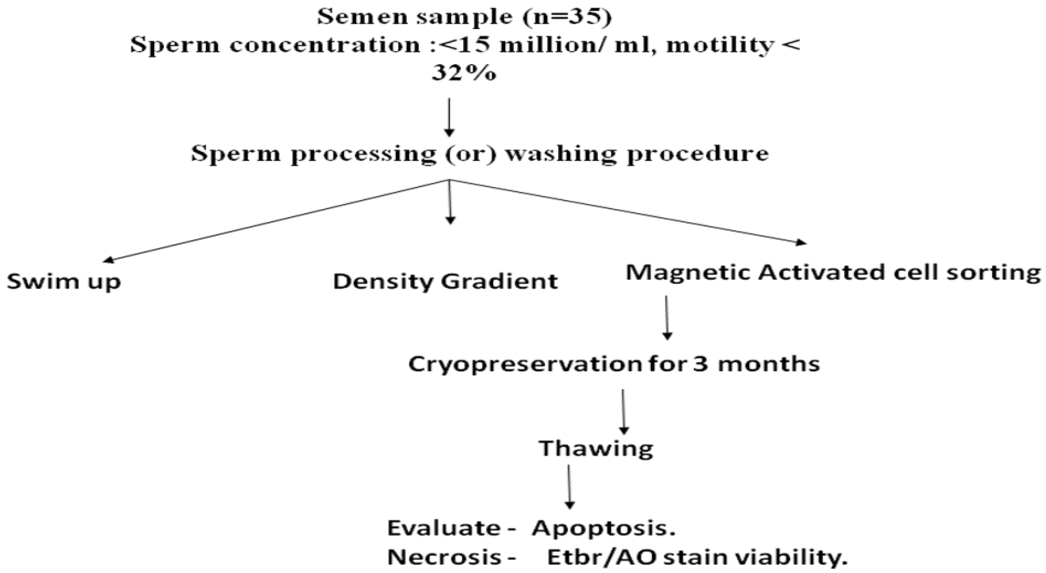
This study focuses on ruling out the best washing method for Oligoasthenospermic patients by analyzing early apoptosis, late apoptosis, Necrosis, and viability using EtBr and Acridine orange stain using methods of Density Gradient, Swim-up & Magnetic Activated methods.

MATERIALS REQUIRED

Sperm wash media (VITROMEDia SAR HEALTH), Magnetic activated cell sorting (Canfrag), HEPES medium, Penicillin/Streptomycin antibiotic solution, and Trypsin-EDTA were purchased from Gibco (USA), EtBr and Acridine orange was purchased from Sigma Aldrich (USA). Fluorescence microscope (NIKON)

METHODS

Semen samples were obtained from 30 Oligoasthenozoospermia patients with progressive motility <32%, who underwent ART procedure in Sumathi Fertility Centre, Madurai between June-October 2021. Informed consent was obtained from all men prior to the study. This research was approved by the institutional review board of Bharath University, Chennai.SBDCH/IEC/02/2017/09



Briefly, the semen sample was subjected to swim up, density gradient, and/or magnetically activated cell sorting method. After washing, the samples were cryopreserved in liquid nitrogen for a period of 3 months. After 3 months Apoptosis, Necrosis and viability were assessed by using EtBr and Acridine orange stain (Figure 1).

Principle

Fluorescent dyes with aromatic amino or guanidine groups, such as acridine orange (AO), interact with nucleotides to emit fluorescence. EtBr molecules intercalate inside the DNA double helix. AO can form complexes with either double-stranded DNA or single-stranded DNA and RNA. One molecule of AO can also interact with one phosphate group of single-stranded DNA or RNA to form an aggregated, or stacked, structure that emits red fluorescence with a maximum wavelength of 650 nm. This fluorescent dye is impermeable through the cell membranes of viable cells and can be used as fluorescent indicated dead cells. Acridine orange is a vital dye and will stain both live and dead cells. Necrotic cells stain orange and have a nuclear morphology resembling that of viable cells, with no condensed chromatin. Ethidium bromide (ETBr) is only taken up by cells when cytoplasmic membrane integrity is lost and stains the nucleus red. EtBr dominates over AO. Thus live cells have a normal green nucleus; early apoptotic cells have a bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus. Ethidium re-emits this energy as yellow/orange light centered at 590 nm. The fluorescence of ethidium bromide in an aqueous solution is significantly lower than that of the intercalating dye

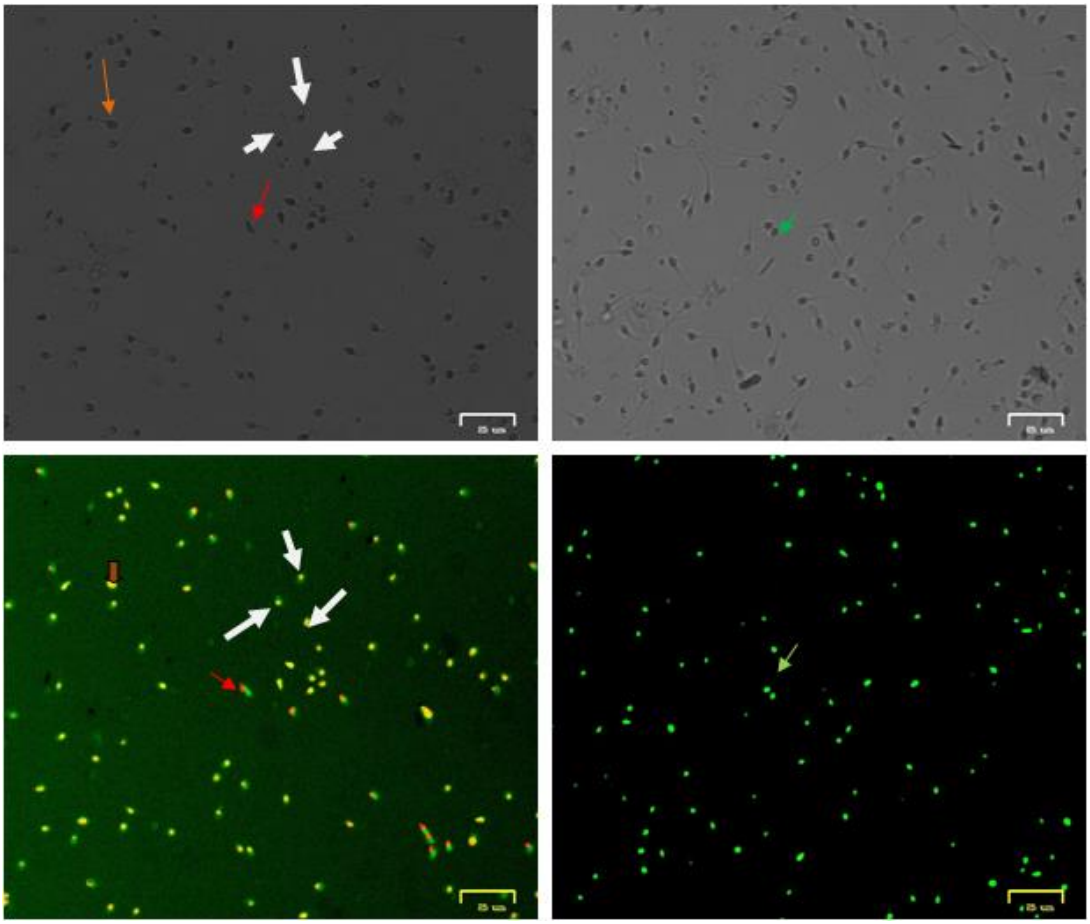
ETBr/AO staining:-

After Thawing $5x10^5$ cells/ml of sperm cells were plated into a 96-well tissue culture plate add 10 µOf 1 mg/ml acridine orange and ethidium bromide were added to the wells and mixed gently. Finally, the plate was kept in laminar flow and evaluated immediately within an hour, and examined at least 1000 cells by a fluorescence imaging system (ZOE, BioRadUSA)

STATISTICAL ANALYSIS

Statistical Analysis was done by using SPSS Version 26. One Way ANOVA test will be used to find the significance between groups. P<0.05 will be considered statistically significant.

Figure 2: Images pertaining to the sperm present after different washing methods



Observations:

The following are the observations seen in Figure 2.

1. Early and late apoptosis is more seen in the Swim-up method Yellowish green-Early apoptosis. Orange-Late apoptosis.

2. Viable cells (green) more in the Density gradient method.

3. Necrosis (red) is more seen in the Magnetic activated cell sorting method.

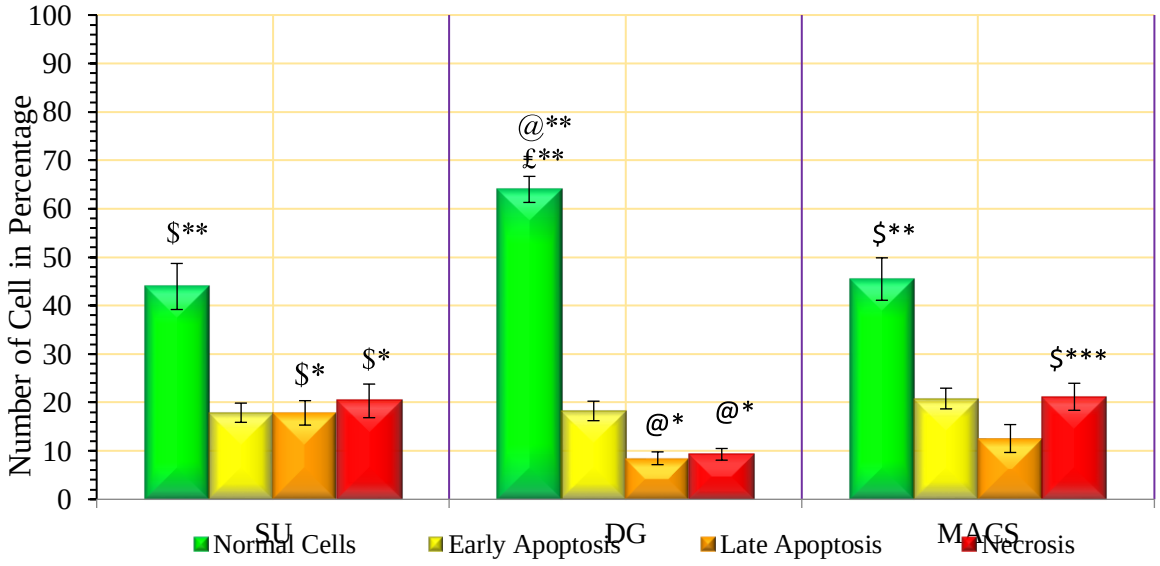
RESULTS

Table 1. Ethidium Bromide and Acridine Orange Stain

Groups	Normal Cells	Early Apoptosis	Late Apoptosis	Necrosis
SU	43.94 ± 4.76 $**	17.88 ± 1.99	17.85 ± 2.52$*	20.33 ± 3.48$*
DG	63.99 ± 2.69@**,£**	18.25 ± 2.01	8.47 ± 1.31@*	9.29 ± 1.20@*,£***
MACS	45.48 ± 4.38$**	20.80 ± 2.14	12.55 ± 2.88	21.17 ± 2.79$***

Table (1) displays the quantitative assessment of different cell types using Ethidium bromide and Acridine Orange Stain. The percentage of fluorescent positive cells is presented as mean ± SEM. Data were analyzed by one-way ANOVA and multiple comparisons between groups were performed by Tukey's test SU-Swim up method; DG - Density Gradient method; MACS - Magnetic Activated cell sorting method. @ - comparison with SU; $ - comparison with DG; £ - comparison with MACS. Statistically significant was achieved at * P<0.05, ** P<0.01 and *** P<0.001.

Ethidium Bromide and Acridine Orange Stain



Conclusion:

The procedure of cryopreservation and thawing is clearly harmful to sperm viability. Simple necrosis or necrosis due to apoptosis could be the cause of the dead cells with high PI permeability. Water freezes as ice during conventional freezing, causing tissue damage, cytoplasmic structures, and even cytoskeleton and genome-related structures to be affected. In many cell types, ETBr and AO staining has been universally regarded as a prediction of DNA damage. The importance of sperm DNA damage in sperm is still up for debate, as it may or may not be linked to male fertility [9]. In the current study, three methods of Density Gradient, Magnetic activated, and Swim up methods are used to analyze the early &late apoptosis, Necrosis and Live cells in the

sample after cryopreservation. The swim-up method had higher early and late apoptosis was seen in it and a Yellowish green color showed early apoptosis and an Orange color was seen with respect to late apoptosis. The viable cells were seen more in the Density gradient method whereas the Magnetic Cell sorting method showed increased levels of Necrosis. The density gradient method shows more viable cells when compared to swim-up and magnetic activated and cell sorting methods. Early and late apoptosis is more seen in Swim up method not suitable for ICSI and IVF methods. The magnetic method shows high necrosis in semen processing and is not suitable for the ICSI method.

DISCUSSION:

The normal, predictable, and rapid return to function of cells after cryopreservation is now the focus of cell-based applications in cell therapy, regenerative and reparative medicine, biobanking, and tissue engineering. With today's tools and methodologies, this is frequently not possible. Continuous improvement in cryopreservation outcomes will rely on the convergence of cellular biology, molecular biology, biophysics, engineering, and cryobiology to overcome this issue. Furthermore, given the expanding body of data that CPAs like DMSO have an impact on the cells, proteome, genome, and structures including the mitochondria, cell membrane, and nucleus, it is clear that successful preservation will necessitate new ways for new definitions of success.

Traditionally, cryopreservation research has concentrated on the incorporation of penetrating cryoprotectants and the management of ice and chemo-osmotic disturbances in order to preserve the structural integrity of cells. New tactics improved preservation outcomes by altering the preservation solution to counteract some of the negative effects of stress that contribute to the initiation of apoptotic and necrotic cell death cascades after thawing. The literature base for understanding and developing novel ways to preserve using an integrated approach is slowly growing. With multiple methods coming up on sperm washing methods and preservation methods, the current study enables researchers to choose the most viable and advantageous. One aspect to be kept in mind by future researchers is that the current research is based on clinical study, however, the practical application in ARTs are to be researched on in detail.

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