

Comparing different sperm separation techniques for ART, through quantitative evaluation of p53 protein

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ABSTRACT

Context: In the last 10 years, assisted reproductive technologies (ARTs) have offered infertile couples an opportunity to complete their reproductive project. However, the high failure rate could be explained with the complex human reproduction system. In ART, the decrease of the success is due to the conditions far from the natural ones. **Aims:** The aim of this study is to evaluate deoxyribonucleic acid (DNA) damage of spermatozoa before and after selection procedures, using a new technique able to quantize sperm DNA damage. **Settings and Design:** They were involved 43 males domiciled permanently in two areas with different Environmental Impact, HEI (high environmental impact) and LEI (Low environmental impact), they are aged between 24 and 31 years with various degrees of dyspermia. **Subjects and Methods:** The 43 males were divided into two groups: 21 in Group A (EIL) and 22 in Group B (EIH). The samples must be aliquoted into parts of 0.5 mL: Group (a) Control, no processing; Group (b) Swim-up (SUP) from semen; Group (c) classic SUP; Group (d) density gradient centrifugation (DGC). All samples were subjected to a quantitative dosage of p53 protein, before and after processing. **Statistical Analysis Used:** For the development of the probability and significance of the data, the Student's *t*-test was used. **Results:** From our data, it emerges that Groups D and B provide a superior quality about motility, vitality, and apoptosis indexes compared to other conventional techniques. In Group B, apoptosis is comparable to Group D, but they have slightly lower about motility and vitality. Group C is the one that has lower parameters than the other techniques. Regarding the evaluation of p53 protein, the results are conflicting with the evaluation of apoptosis; in fact, in Group D, the values are significantly higher than the other techniques. **Conclusions:** Sperm separation is an important moment in ART techniques. From our data, it emerges a greater fragility of DNA in the male spermatozoa who reside permanently in areas with high environmental impact.

KEYWORDS: GDC, EIL.EIH, P53, swim-up

INTRODUCTION

Considering both primary infertility and secondary infertility, a systematic review^[1] reports that infertility affects 15% of couples in developed countries. In the past 10 years, assisted reproductive technologies (ART) have offered infertile couples an opportunity to complete the

reproductive project. However, these technologies do not offer a safe result (baby born alive)^[2] and consequently many couples' experience is a failure and they have to face several attempts to complete their desire.

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From the birth of Louise Brown on July 25, 1978, and the subsequent race toward ART, biologists and andrologists have been urged to improve sperm separation techniques. The first cases of *in vitro* fertilization (IVF) were performed to treat female infertility (tubal pathology), but later it became evident that male partner also reported severe dysspermia. This has induced to search for sophisticated methods, to select functionally competent spermatozoa. The complexity of the reproductive machine can explain the high failure rate,^[3] but also the conditions, far from the natural ones, contribute to decrease the success of the ART. *In vitro* manipulation of gametes and embryos can expose them to various adverse conditions.^[4] Under natural conditions, spermatozoa suffer an intense quantitative and qualitative selection process. Potentially fertile spermatozoa are separated by nonvital spermatozoa, during the passage in the female genital tract, by active migration through the cervical mucus.^[5] During this process, mobile spermatozoa are selected and then they are subjected to an important physiological modifications, including capacitation, which is a fundamental prerequisite for the acrosome reaction,^[6,7] ensuring that only the best spermatozoa reach the oocyte and can start the embryo development process, under the best conditions.^[5]

The ideal sperm separation technique should meet the following requirements:

- Be fast, easy to perform, and cheap
- Isolate many mobile and functionally competent spermatozoa, as possible
- Do not cause damage to the sperm or alterations that affect the fertilizing capacity
- Eliminate not vital spermatozoa, including leukocytes and bacteria
- Eliminate toxic substances, such as decapacitation factors and reactive oxygen species (ROS)
- Allow the preparation of ejaculate volumes above the norm (hypospermia).

Because none of the available methods can satisfy all these requirements, a variety of sperm separation techniques are compulsory in laboratory practice, to obtain an optimal yield of functionally competent spermatozoa, for assisted fertilization. The first available sperm separation methods were based on one or two washing procedures only, with consequent suspension of male germ cells.^[8,9] Then, Mahadevan and Baker^[10] in 1984 described a single wash, followed by a swim-up (SUP) procedure by cellular pellet (precipitate). From these first attempts, more sophisticated methods were developed. Currently, the most used techniques in medically assisted procreation centers are SUP with its variants and density gradient centrifugation (DGC).^[8,10,11]

Although numerous studies have been published on the efficacy of these techniques, there is not enough evidence to recommend one of them.^[12]

In recent years, comparative studies on sperm preparation methods have essentially evaluated the results, such as recovery rates and conventional sperm parameters (number, motility, and morphology).^[13-15] Only lately, researchers have focused on rating molecular parameters, such as sperm deoxyribonucleic acid (DNA) damage or apoptosis, to better evaluate these different separation techniques.^[16] Spanò *et al.*^[17] in 1999 demonstrated that sperm separation with the SUP technique did not cause a significant change in the structure of the spermatozoa chromatin. The same result was achieved by Younglai *et al.*^[19] in 2001. Moreover, Zini *et al.*^[18] in 2000 demonstrated that the percentage of spermatozoa with denatured DNA was significantly reduced in the spermatozoa treated with the SUP technique, but not in spermatozoa treated with density gradient centrifugation (DGC) technique.

In contrast, Donnelly *et al.* in 2000 and Marchetti *et al.* in 2002 reported that the mobile spermatozoa prepared with density gradient centrifugation (DGC) showed a better mitochondrial membrane potential and a lower DNA fragmentation, generated lower amounts of ROS, and were more viable compared to sperm from untreated seminal fluid.^[20,21] Furthermore, according to the results of Sakkas *et al.*^[22] in 2000, a significant decrease in the percentage of spermatozoa with DNA damage is shown in the use of DGC techniques, while in the use of the SUP method, the recovered spermatozoa show no significant percentage of improvement.

On the other hand, some studies about apoptosis of spermatozoa prepared with both SUP^[23,24] and DGC^[25,26] report conflicting results. DGC technique selects spermatozoa with better motility^[27] and better morphology and maturity^[28] from the entire seminal fluid; however, recent evidence indicates that this technique can increase the levels of sperm DNA fragmentation (sDF),^[29,30] a parameter that has a negative impact on the outcome of assisted procreation (ART).^[24] In particular, DGC increases sDF in about 50% of seminal fluids treated for IVF/intracytoplasmic sperm injection (ICSI),^[30] reporting a pregnancy rate below 50%.^[30]

In this contest, it seems important to identify seminal fluids, in which there is an increase in sDF during selection with DGC and to evaluate alternative sperm selection. Waiting for scientific feedback on the actual damage caused by DGC, many centers make use of old SUP technique.

Currently, there are no studies indicating a significant increase in sDF with this procedure;^[31] the studies only

report average values of sDF before and after selection, without evaluating an effective rate of pregnancy.

As previously highlighted, selection procedures improve motility and eliminate spermatozoa that are mainly dead with fragmented DNA.^[32] The aim of this study is to evaluate the DNA damage of spermatozoa before and after selection procedures (DGC, classic SUP, and SUP from semen), comparing the data with pretreatment values (control), using a new technique able to quantize sperm DNA damage. The reference technique is that proposed by Raimondo *et al.*^[33] in 2014: the quantitative evaluation of p53 protein on sperm DNA, corrected with sperm concentration. An immunoenzymatic method (enzyme-linked immunosorbent assay [ELISA]) is used, which meets laboratory requirements for precision, reliability, and repeatability.

SUBJECTS AND METHODS

For this study, we considered 43 males in the period between January 2015 and December 2018.

They were divided into two groups: Group A, 21 males domiciled permanently in an area with low environmental impact (southern area of Salerno, Italy) and Group B, 22 males domiciled permanently in an area with high environmental impact (northern area of Napoli “land of fires”, Italy).

The males considered are between 24 and 31 years old and the volume of their ejaculates varies from 2.6 to 4.6 mL, respectively; moreover, they present various degrees of dyspermia, equally represented in the two groups.

Individuals spermatic evaluation in examination has been detected with spermogram,^[33] adopting standard analysis criteria according to the World Health Organization (WHO) manual fifth edition – 2010 (WHO laboratory manual for the examination and processing of human semen).

Makler counting (Makler Counting Chamber, Sefi Medical Instruments Ltd.) has been utilized for the evaluation of nemaspermic concentration, expressed in mL, as well as in the study of nonsperm cellular component (leukocytes, red blood cells, and germ line cells).^[34]

The males involved did not consume cigarettes, alcohol, drugs and also did not have any chronic pathology in 6 months prior to collection of seminal fluid. These individual were also not exposed to environmental toxins^[35-37] and did not have pathological varicocele at preliminary examination with Doppler.^[38-40]

The participants signed the informed consent form for the processing of personal and sensitive data, as well as

of genetic and biological sample collection in compliance with the applicable laws.^[41,42] The signed informed consent was obtained from the participants of the study. The procedures followed were in accordance with the ethical standards of experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 2000.

The processing steps of semen samples were carried out after liquefaction, between 30 and 45 min after ejaculation. The samples were aliquoted into five parts of 0.5 mL, four of which were immediately processed and the fifth part was frozen to -20°C for future investigations.

The four aliquots are treated as follows:

- Group A: Control, no processing
- Group B: SUP from semen
- Group C: Classic SUP
- Group D: DGC.

Preparation methods of seminal fluid

Group A: Control

On control, the sample was performed a quantitative evaluation of p53 protein, at time 0 and 60 min. During this time, the seminal fluid does not undergo any treatment; it is placed in an incubator at 37°C and 5% of CO_2 , in a 15 mL Falcon tube.

Group B: Swim-up from semen

An aliquot of seminal fluid is placed under 300 μL layer of a culture medium (Quinn's, SAGE, USA). The sample contained in a 15 mL Falcon tube is placed with an inclination of 45° , to increase the contact surface between the seminal fluid and the culture medium, for 30 min in an incubator at 37°C and 5% of CO_2 , in a 15 mL Falcon tube. The supernatant fraction is removed and sent to the subsequent evaluation process.^[43]

Group C: Classic swim-up

An aliquot of 0.5 mL of whole sperm is mixed with 1.0 mL of spermatozoa culture medium, complemented with 0.1% of human serum albumin (Sigma-Aldrich, St. Louis, Catalog – A1653), incubated at 37°C , in a 15 mL Falcon tube and centrifuged at 200 g for 8 min. The supernatant is removed and the precipitate (pellet) is mixed with 1.0 mL of culture medium and then centrifuged at 100 g for 45 min. Hence, after eliminating the supernatant, 300 μL of culture medium is gently stratified on the final pellet. The tube is placed, with an inclination of 45° , to increase the contact surface between the seminal fluid and the culture medium, for 30 min in an incubator at 37°C and 5% of CO_2 .^[13] The supernatant fraction is removed and sent for subsequent evaluation process.

Group D: Density gradient centrifugation

The 80/70 gradients (Pureception, SAGE, USA) were placed in 15 mL Falcon tubes, followed by the stratification of 0.5 mL of whole ejaculate and then centrifuged at 200 g for 20 min.

The gradient is removed and the pellet is washed twice ($200\text{ g} \times 5\text{ min}$) with 1.0 mL of culture medium. The final pellet is overlaid with 300 μL of culture medium and it is placed in an incubator at 37°C and 5% of CO_2 , for 30 min.^[44,45] The supernatant fraction is removed and sent for subsequent evaluation process.

All samples are subjected to a quantitative dosage of p53 protein, in relation with the number of spermatozoa. The method was presented in one of our previous studies and it is shown below.

Separation of sperm from seminal fluid

To perform the isolation of sperm from semen, the Differex System™ for use with the Differex Magnet™, and DNA IQTM System—Small Sample Casework Protocol kits were used (Promega Corporation, Madison WI, USA). In 1985, Gill *et al.*^[46] developed a method to separate spermatozoa from epithelial cells in a sample of human semen. The separation protocol reported below has been developed for this project. It requires about 150 min to obtain the complete separation and purification of the sperm DNA.

The number of sample and reagents quoted has been calculated for a single sample and a single experiment in the following protocol.

One hundred microliters of sample was placed in a 1.5 ml tube with 400 μL of digestion solution containing 6 mL of diluted Proteinase K and 364 μL of digestion buffer. The tube was vortexed for 30 s at 14,000 rpm, incubated for 90 min at 56°C , and then centrifuged for 10 min at 14,000 rpm in a microcentrifuge at room temperature, not before having marked the position where the pellet would form.

Then, 3.5 μL of DNA IQTM resin was added in the opposite position of the pellet and the tube was placed on Differex Magnet™, so that the resin, attracted by the magnet, would coat the pellets. The yellow liquid layer, containing epithelial cells, was then removed.

The sample was then washed 3 times with 500 μL of nuclease-free water and the last washing volume was not removed.

The tube was centrifuged again at 14,000 rpm for 10 min and 3.5 μL of DNA IQTM resin was added in a position opposite the pellet and positioned in Differex Magnet™, so that the resin would coat the pellets. After

three washes, a further 500 μL of nuclease-free water together with 100 μL of separation solution was added, so that the resin would coat the pellets. The washing and separation solutions were then removed and the pellet was resuspended by adding 400 μL of 0.9% sodium chloride solution.

To extract the sperm DNA, 250 μL of lysis solution (containing 2.5 μL of DTT and 252.5 μL of Lysis Buffer) was added to the tube, which was then vortexed for 3 s at high speed and incubated for 5 min at room temperature. After having vortexed again for 3 s, the tube was positioned in the Differex Magnet™, so that the separation would occur instantaneously and the supernatant was then removed and stored in another tube (“lysed sample”).

Then, 100 μL of lysis solution was added and the tube was removed from the Differex Magnet™. After vortexing for 2 s, the tube was put back in Differex Magnet™ and the entire lysis solution was eliminated.

To perform the washing, 100 μL of wash buffer was added after removing the tube from the Differex Magnet™ and the sample was vortexed for 2 s at high speed.

Once the tube was repositioned in Differex Magnet™, the entire wash solution (containing 500 μL of wash buffer, 250 μL of isopropyl alcohol, and 250 μL of ethanol) was eliminated.

The washing procedure was repeated 3 times.

The resin was allowed to air-dry for 5 min, leaving the tube with the cap open.

Subsequently, 100 μL of elution buffer was added (10 mM Tris pH 8.0 and 0.1 mM EDTA), and the tube was vortexed for 2 s and incubated for 5 min at 65°C and vortexed again and placed immediately on Differex Magnet™. The solution containing the DNA was carefully transferred in a new tube (“lysed sample and refined DNA”).

Quantitative p53 dosage with enzyme-linked immunosorbent assay

A direct and quantitative ELISA assay was used to measure p53 (DuoSet IC, Human Total p53 R and D Systems Inc., Minneapolis, MN, USA).

Briefly, 100 μL of the capture antibody, appropriately diluted, was pipetted into each well of a 96-well microplate, which was then sealed and incubated overnight at room temperature. The next day, the plate was washed 3 times with 400 μL wash buffer (0.05% Tween 20 in PBS, pH 7.27.4, filtered at 0.2 μL).

Each well was blocked with the addition of 300 μL of stop solution (Sample Diluent Concentrate: 5X PBS,

Table 1: Minimum, Maximum and average values of Group A °Before and after sperm separation technique, *60 min after the first evaluation of p53 § single control assessment

	Value for groups: p53 ng/millionspermatozoa					
	Group A					
	Before °			After °		
	minimum	maximum	average	minimum	maximum	average
Group (a) “control”	0,63	2,01	X _{1,21} §	1,12	2,54	X _{1,37} *
Group (b) “Swim-up from semen”	0,63	2,01	X _{1,21} §	1,55	3,01	X _{1,49}
Group (c) “classic Swim-up”	0,63	2,01	X _{1,21} §	2,46	4,65	X _{2,98}
Group (d) “DGC”	0,63	2,01	X _{1,21} §	3,26	7,12	X _{4,23}

Table 2: Minimum, Maximum and average values of Group B °Before and after sperm separation technique, *60 min after the first evaluation of p53 § single control assessment

	Value for groups: p53 ng/millionspermatozoa					
	GroupB					
	Before °			After °		
	minimum	maximum	average	minimum	maximum	average
Group (a) “Control”	2,24	4,56	X _{3,31} §	3,17	7,36	X _{4,97} *
Group (b) “Swim-up from semen”	2,24	4,56	X _{3,31} §	3,85	8,98	X _{5,87}
Group(c) “calssicSwim-up ”	2,24	4,56	X _{3,31} §	4,02	9,74	X _{8,27}
Group (d) “DGC”	2,24	4,56	X _{3,31} §	7,31	16,67	X _{11,61}

Table 3: Minimum, Maximum and average values of Groups A and B, show the “P values” about groups and techniques used. °Before and after sperm separation technique, *60 min after the first evaluation of p53 § single control assessment

Groups of samples	p53 ng/millions spermatozoa				p
	Group A		Group B		
	Before °	After °	Before °	After °	
Group (a) “control”	X _{1,21} §	X _{1,37} *	/	/	N.S.
Group (a) “control”	/	/	X _{3,31} §	X _{4,97} *	N.S.
Group (b) “Swim-up from semen”	X _{1,21} §	X _{1,49}	/	/	N.S.
Group (b) “Swim-up from semen”	/	/	X _{3,31} §	X _{5,87}	N.S.
Group (c) “classic Swim-up”	X _{1,21} §	X _{2,98}	/	/	N.S.
Group (c) “classic Swim-up”	/	/	X _{3,31} §	X _{8,27}	p<0,05
Group (d) “DGC”	X _{1,21} §	X _{4,23}	/	/	p<0,05
Group (d) “DGC”	/	/	X _{3,31} §	X _{11,61}	p<0,001

5 mM EDTA, and 2.5% Triton X100). The plate was then incubated at room temperature for 2 h. Extraction and washing steps were repeated. The standards were prepared by diluting in IC Diluent # 4 (1 mM EDTA, 0.5% Triton X100 in PBS, pH 7.2–7.4.) and using IC diluent #4 like standard zero.

Then, 100 µL of sample or standard was added (“lysed and purified DNA” cell preparations), and the plate was sealed and incubated for 2 h at room temperature. After the incubation, extraction and washing were repeated and 100 µL of detection antibody (total p53 detection antibody), appropriately diluted, was pipetted into each well.

The plate was sealed and incubated for 2 h at room temperature. After the incubation, extraction and

washing were repeated. One hundred microliters of StreptavidinHRP was then added and the plate was incubated for 20 min at room temperature. After the incubation, extraction and washing steps were repeated and 100 µL of substrate solution (1:1 mixture of Reagent A and Reagent B) was added to each well and the plate was incubated for 20 min at room temperature. Finally, 50 µL of stop solution was added to each well. The optical density was analyzed using a microplate reader set at 450 nm with a software that automatically calculated the concentrations expressed in pg/100 µL.

RESULTS

The minimum, maximum, and average values for Groups A and B, with relative techniques used, are shown in Tables 1 and 2.

Statistical analysis

Pearson's correlation coefficient "*r*" (a dimensionless index ranging between -1.0 and $+1.0$, which reflects the extent of a linear relationship between two data sets) was used for the statistical analysis of the groups. Fisher's transformation "*x*" was used to perform a hypothesis test on the correlation coefficient. Finally, for the development of probability and data significance, the Student's *t*-test was used. These statistical calculations were performed using Microsoft Excel 2008 (Microsoft Corporation, Redmond WA, USA).

DISCUSSION

In this study, p53 protein concentrations changed significantly in the two groups and with the different methods used [Tables 1 and 2]. The average percentage of apoptotic spermatozoa is evaluated with acridine orange test,^[47] In the samples treated with the classic SUPmethod, Group C, the average percentage of apoptotic spermatozoa is significantly higher than samples processed with density gradient (GDC), Group D, and "SUP from semen", Group B. The lowest percentage of apoptotic spermatozoa found in Group B and Group D suggests that these methods allow to get supernatant with fewer spermatozoa with fragmented DNA. The negative association between spermatozoa apoptosis and fertilization rate has been documented in numerous studies.^[48,49] The selection of nonapoptotic spermatozoa should be one of the most important requirements to obtain optimal conception rates in ART;^[49] it is certain that an important parameter is choose a optimal separation method, that better emulates the natural selection.

Sperm separation is an important moment in ART techniques. From our data, it emerges that the methods with density gradient centrifugation (Group D) and the SUP from semen (Group B) provide a superior quality about of motility, vitality, and apoptosis indexes compared to other conventional techniques. In Group B, apoptosis is comparable to Group D, but they are slightly lower about motility and vitality. Group C is the one that has lower parameters than the other techniques. About the evaluation of p53 protein, the results are in contrast with the evaluation of apoptosis; in fact, in Group D, the values are significantly higher than the other techniques.

About the meaning of presence of p53 protein, several authors hypothesized that it has an important role in oocyte maturation in the development of blastocyst and implantation of the embryo in reproduction.^[50] Blastocysts obtained from *in vivo* fertilization have low concentrations of p53 protein, whereas p53 expression is higher in embryos obtained from IVF.

These observations suggest that embryonic culture leads to accumulation of p53 protein transcription activity in blastocyst and it may be one of the reasons causing delayed embryo growth.^[51]

Human embryos generated by ICSI have elevated nuclear p53 expression, associated with delayed embryonic development.^[52]

CONCLUSIONS

It emerges a more complex p53 protein role, which is different from the only control of sperm DNA integrity, and it is hypothesized that p53 could control timing and embryonic development.

About the differences in concentrations between the various methods, in the groups with different environmental impact, the data show a greater fragility of DNA in spermatozoa of subjects who live permanently in areas with high environmental impact (Group B) [Table 3].

One of our ongoing studies is about telomere length to demonstrate effective sperm DNA fragility, in participants who permanently live in areas with different environmental impacts.

Our study is well integrated with the importance of having an objective and repetitive data, preliminary to conception both *in vivo* and *in vitro*. Certainly, further experiments will be needed to confirm the hypothesis of this study; moreover, it would also be interesting to compare with pregnancy rates.

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Conflicts of interest

The authors declared that they have no conflict of interests. They have not received financial rewards for the execution of the procedure or any sponsorship. The author declares that this study was made in accordance with the Italian principles governing research.^[40,41]

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