

# HUMAN SPERM PRESERVATION USING FREEZE-DRYING An EXPERIMENTAL LABORATORY STUDY

## *Studi Laboratorium : Preservasi Sperma Manusia Dengan Menggunakan Teknik Freeze-Drying*

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### *Abstrak*

**Latar Belakang :** Terdapat beberapa teknik preservasi sperma yang masing-masing memiliki kelebihan dan kekurangan. Salah satu alternatif tekniknya adalah dengan teknik pengeringan. Teknik ini bersifat lebih murah, mudah, dan praktis. Teknik freeze-drying masih merupakan studi empiris yang harus dibuktikan kebenarannya.

**Tujuan:** Penelitian ini bertujuan mengevaluasi kemungkinan aplikasi teknik *freeze-drying* untuk preservasi sperma manusia dan menentukan pengaruh proses yang diikuti penyimpanan selama 7 hari pada suhu 4°C terhadap integritas DNA spermatozoa.

**Metoda:** Pada penelitian ini digunakan *Posttest Only Control Group Design*. Populasi adalah sperma pria dewasa berusia 21-40 tahun dengan criteria normozoospermia menurut standard analisis semen WHO. Besar sampel adalah 12, diperoleh dengan cara *simple random sampling*. Integritas DNA spermatozoa dari sampel semen segar, segera setelah freeze-drying dan yang disimpan selama 7 hari pada suhu 4°C, diuji dengan *Comet assay*. Hipotesis diuji dengan Manova.

**Hasil:** integritas DNA spermatozoa kelompok sperma yang diberi perlakuan *freeze-drying* diikuti penyimpanan selama 7 hari pada suhu 4°C secara signifikan lebih rendah dibandingkan dengan kelompok sperma segar atau yang hanya diberi perlakuan *freeze-drying* ( $p < 0,01$ ).

**Kesimpulan:** Tidak ada perbedaan integritas DNA spermatozoa yang signifikan antara kelompok sperma segar dengan sperma yang diberi perlakuan *freeze-drying* ( $p > 0,05$ );

**Kata kunci:** *Comet assay, Integritas DNA, Teknik Freeze-drying*

### **Abstract**

**Background:** Several techniques of sperm preservation has developed and each sperm preservation techniques have advantages and disadvantages. One alternative technique for sperm preservation is drying technique. The method of drying has an advantage because the cost is relatively cheap, easy and practical. Until now the possibility of application of freeze-drying techniques for human sperm preservation new little researched and freeze-drying technique is still the empirical method.

**Objective** The objective of this study was to evaluate the application of freeze-drying technique on human sperm preservation and to determine the influence of freeze-drying process and 7 days storage under 4° C temperature on human sperm DNA integrity.

**Method:** The Posttest Only Control Group Design was used in this study. Population was sperm of adult male over 20 years old. Population of adult male sperm was over 20 years old. The sperm should be in normozoospermia category and was obtained with consideration on WHO method. The sample size was 12, obtained with simple random sampling method.. The sample size was 12, obtained with method of simple random sampling. Comet assay was used to determine DNA integrity before freeze-drying, immediately after freeze-drying and 7 days after freeze-drying storage. Manova was used to test the hypothesis.

**Result;** there was very significant difference in DNA integrity between fresh group or freeze-dried group compared with freeze-dried +7 days storage under 4° C temperature ( $p < 0,01$ ).

**Conclusion:** No significant difference in DNA integrity between fresh group and freeze-dried group ( $p > 0,05$ ).

**Keywords :** *Comet Assay, DNA integrity, freeze-drying technique*

## INTRODUCTION

Until recently, several techniques of sperm preservation has developed, such as liquid state sperm preservation, frozen sperm preservation state, other alternative techniques, for example freezing without cryoprotectants and drying. Each sperm preservation techniques have advantages and disadvantages. Storage frozen technique is the most popular nowadays and the principles and practice has been tested extensively (Bwanga 1991, Hammersted & Graham 1992, Hammersted et al. 1990, Holt 2000, Salamon & the Maxwell 1995, Watson 1995 cited from Pangestu 2000).

According to this method of cells (in this case sperm) were frozen and stored at a temperature of  $-196^{\circ}\text{C}$  in liquid nitrogen. However, this technique has several weaknesses which have yet to be resolved, namely higher costs, because the liquid nitrogen tank is relatively expensive and once a week to fill the tank of liquid nitrogen is reduced as a yawn. Impractical, because of a relatively large tank of liquid nitrogen and its heaviness making it difficult to transport and the lifetime is only about 5-10 years. Potential accidents caused by splashes and spills of liquid nitrogen could happen. The storeroom floor where the tank stored should have special protection and the nitrogen should have kept in a specific temperature is  $-196^{\circ}\text{C}$  in order to remain liquid. Some countries already prohibit the transport of liquid nitrogen without a permit or a special truck. (Pangestu 2002)

One alternative technique for sperm preservation is drying technique. This technique has proven very useful and widely used for biological products, clinical, food preservation, pharmaceuticals and agriculture. The method of drying has an advantage because the cost is relatively cheap, easy and practical (Pangestu 2002).

Several attempts to freeze dried (*freeze-drying*) of animal and human sperm is reported to have succeeded. Spermatozoa, especially of mammals can be stored in dry conditions without losing the potential to

fertilize and produce normal embryonic development. Must be considered in the process of drying is to be free of oxygen, because oxygen will trigger the formation of reactive oxygen compounds that can damage the cell membrane to the core level. Therefore efforts should be made to minimize the entry of oxygen into the sperm storage (Pangestu cited from Gaylord 2000).

Until now the possibility of application of *freeze-drying* techniques for human sperm preservation new little researched and *freeze-drying* technique is still the empirical method. Knowledge of the genetic stability during storage in various media is also still unclear. So still need to do research on *freeze-drying* techniques for human sperm preservation, especially, because if this technique is successfully applied, the constraints of storage with liquid nitrogen, high costs and transportation can be handled.

Even the sampling and storage is not necessary in clinical IVF (*In Vitro fertilization*), be conducted in public health institutions at affordable peripheral and can be stored at home. Besides saving costs, it could also prevent cross-contamination between specimens. Transportation to the IVF clinic will be conducted if the ICSI is also easier and cheaper (Pangestu M cited from Gaylord 2000, Ward MA et al. 2003).

This research will use the technique of *freeze-drying* by freezing sperm at  $-20^{\circ}\text{C}$  and then dried with a *freeze-dryer*. Sperm is placed in a vial glass, rubber lids that are not toxic. Integrity DNA was evaluated on fresh sperm, the sperm treated with *freeze-drying* and sperm treated with *freeze-drying* + storage at  $4^{\circ}\text{C}$  for 7 days.

After *freeze-drying* must be followed by ICSI procedure, the evaluation of DNA integrity is very important (Morris et al. 2002). *Comet assay* used to assess the integrity of the DNA because it relatively simple, rapid and sensitive and can evaluate the integrity of the DNA of single cells. (Hartman & Speit 1994 adapted from Rojas et al. 2000, Rojas et al. 2000, Singh et al. 1998, Hartman & Speit 1994 adapted from Rojas et al. 2000).

## MATERIALS AND METHODS

Sperm adult male population is aged t21 years, with the results of sperm analysis normozoopermia. The sample is male sperm adults aged t21 years, with the results of sperm analysis normozoopermia taken by *simple random sampling* of clinic visitors Andrology dr Soetomo to obtain  $n = 12$ . Sample size is determined by the formula:  $n = [(Za + Z b)^2 / (x_c - x_t)^2] SD^2$  where the observation of pairing:  $SD^2 = (x_c - x_t)^2 SD^2 = (x_c - x_t)^2$  (Note :  $2 a = 1.96$  and  $2 b = 1.25$  ) so that  $n = (1.96 + 1.25)^2$  and the result  $n = 10.3 \gg 12$  people

The research design used was the posttest charge control group design:

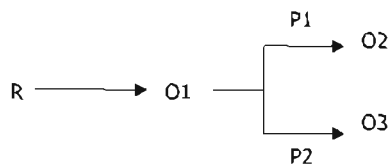


Figure 1. The posttest charge control group design

R is a random sampling; O1 is the evaluation of sperm DNA integrity by *Comet assay* performed on fresh sperm. P1 is the treatment of *freeze-drying*. O2 is the evaluation of sperm DNA integrity by *Comet Assay* in sperm after *freeze-drying* treatment. P2 is the treatment of *freeze-drying* which continued storage at  $4^{\circ}C$  for 7 days. O3 is the evaluation of sperm DNA integrity in sperm with the *Comet assay* after *freeze-drying* treatment, which continued storage at  $4^{\circ}C$  for 7 days

Data analysis methods that we used are descriptive, to know the general results of the

study and determine the average value and the intersection of raw data obtained in order to calculate the normality of data and further statistical tests. Normality test data, to test the normality distribution of data used *Kolmogorov Smirnov One-Sample Test* and Inferential statistical tests. Scale is the ratio of the data obtained, with a normal data distribution; the tests used *Multivariate Analysis of Variance* for more than one dependent variable. The research was conducted at Andrology Clinic dr. Soetomo, and *Human Genetic Laboratory, Tropical Disease Centre, Airlangga University, Surabaya*.

## RESULT AND DISCUSSION

Sperm DNA integrity assessment conducted by two people and is based on visual scores (*visual scoring*) on the image of the comet was observed on *slides* under a fluorescence microscope. Assessment with a score of his visual equivalent to quantitative computer analysis of comet images (*computer image analysis*). Results of total visual score on a *slide* and then projected on the graph so obtained value of the percentage of sperm DNA fragments that existed at the tail of a comet (Collins 2000).

Comet images that appear on the microscope are classified into five classes, namely: class 0, class 1, class 2, grade 3 and grade 4, a row starting from the comet does not form (either DNA integrity) to which all of his DNA fragmented. Was calculated as the three kinds of dependent variables in this research, namely: the percentage of comets, the visual score, and percentage of tail DNA

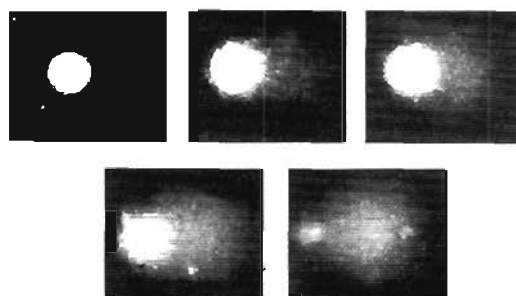


Figure 2. Guidelines for visual scoring of comet images which appears at the fluorescence microscope (AR Collins 2000)

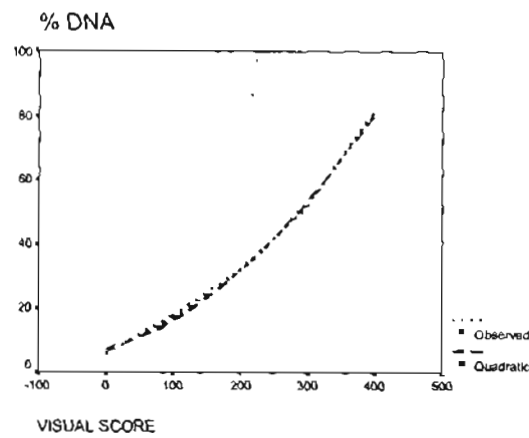


Figure 3. Graph quadratic regression relationship with *Tail% DNA* Visual score (calculated based on the graph Collins 2000)

Table 1. Visual observations and conversion percentage of comet tail DNA on fresh semen samples

No Sample	% Comet	Visual Score (per 100 cell)	% DNA Tails
1.	100.00	140	21.92
2.	63.33	112	17.96
3.	57.04	102	16.65
4.	69.05	126	19.88
5.	73.08	135	21.18
6.	68.40	110	17.69
7.	64.47	131	20.59
8.	66.67	115	18.36
9.	58.33	109	17.56
10.	100.00	150	23.45
11.	58.33	75	13.44
12.	68.75	107	17.30
Mean	70.6208	117.67	18.8307



Figure 4. Image of comet samples of fresh sperm

Table 2 . Visual observations and conversion percentage of comet tail DNA in sperm samples after *freeze-drying*

No Sample	% Comet	Visual Score (per 100 cell)	% DNA Tails
1	90.57	147	22.98
2	81.06	115	18.36
3	82.50	95	15.78
4	75.00	150	23.45
5	70.00	141	22.07
6	74.12	121	19.18
7	90.57	147	22.98
8	78.03	154	24.08
9	71.57	119	18.90
10	94.44	173	27.20
11	88.24	88	14.93
12	85.71	111	17.82
Mean	81.8175	130.08	20.6445

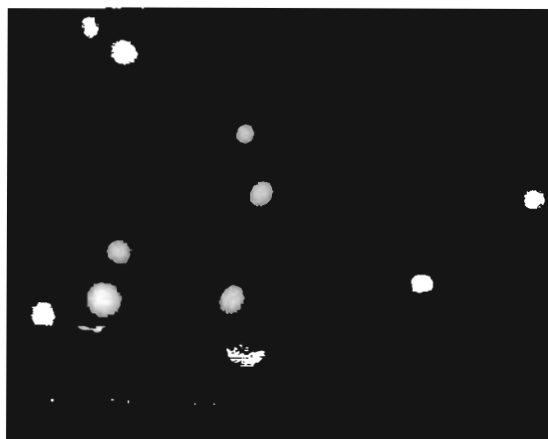


Figure 5 . Image of comet sperm sample post *freeze-drying*

Tables 3 . Visual observations and conversion percentage of comet tail DNA on sperm samples and storage at 4°C for 7 days

No Sample	% Comet	Visual Score (per 100 cell)	% DNA Tails
1	100.00	250	42.05
2	100.00	267	45.81
3	92.86	168	26.36
4	88.57	225	36.84
5	91.67	236	39.08
6	91.67	208	33.51
7	100.00	258	43.80
8	92.31	213	34.47
9	83.86	204	32.75
10	100.00	325	59.94
11	90.00	157	24.56
12	100.00	250	42.05
Mean	94.2450	230.08	38.4335

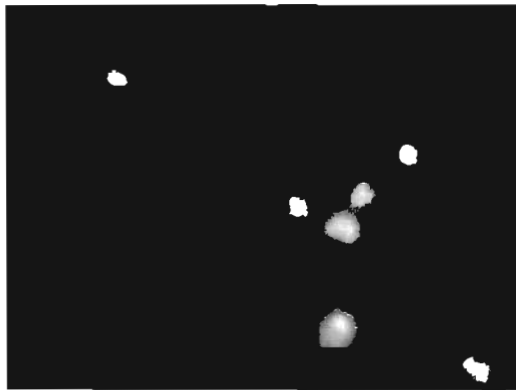


Figure 6. Comet image on sperm samples post *freeze-drying* and storage at 4° C for 7 days

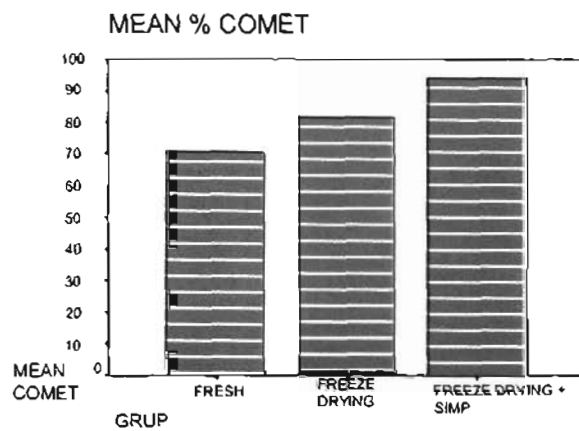


Figure 7. Mean% Comet According to Treatment Group

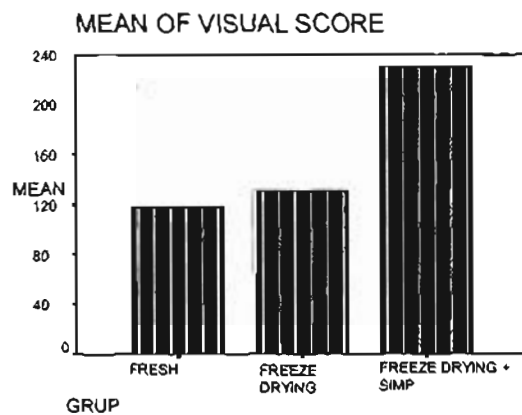


Figure 8. Mean Visual Score According to Treatment Group

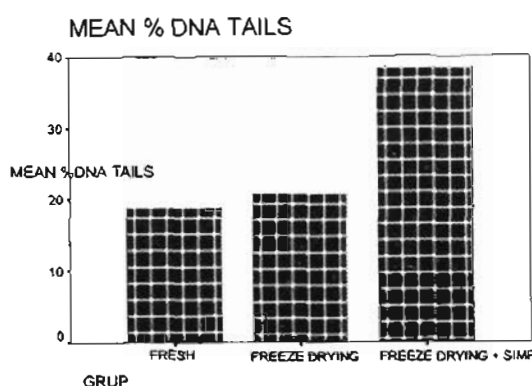


Figure 9. The mean% DNA tails According to Treatment Group

Table 4. Mean and Standard % Deviation Comets, and Visual Scores % DNA tails by treatment group

Report				
Group		% COMET	Visual score	% DNA Tails
Fresh	Mean	70.6208	117.67	18.8307
	SD	14.5739	20.10	2.7084
	N	12	12	12
FREEZE DRYING	Mean	81.8175	130.08	20.6445
	SD	8.1727	25.76	3.6963
	N	12	12	12
FREEZE DRYING + Keep	Mean	94.2450	230.08	38.4335
	SD	5.5808	45.39	9.4651
	N	12	12	12

From a study, the data obtained three variables depend on three groups and treatment groups. Prior to conducting further statistical test, first test for normality with the *One-Sample Kolmogorov-Smirnov test* to determine normality of data distribution of each variable in the three treatment groups. Normality test results showed that the distribution of the data obtained everything was normal ( $p > 0.05$ ). Manova test (*Multivariate Analysis of Variance*) is used to test the three variable data in three treatment groups. The result is: there are significant differences ( $p < 0.01$ ) for the three types of variables in the three treatment groups.

Single Variable Analysis of Variance Test (Univariant) was conducted to examine differences in each variable on the three treatment groups. The test gave the following results: there is a different percentage of the comet is very significant in the three treatment groups ( $p < 0.01$ ), there are differences in visual scores highly significant in the three treatment groups ( $p < 0.01$ ), there

is also differences percentage of DNA tail that is very significant for the three treatment groups ( $p < 0.01$ ).

Further tests conducted is LSD (*Least Significant of Difference*), to assess the degree of difference of each variable in each treatment group. The results obtained are: for the variable percentage of comets, there is significant difference between the fresh group with *freeze-drying* group ( $p < 0.05$ ), there are significant differences among fresh groups with a *freeze-drying + storage* group ( $p < 0.01$ ) and no difference very significantly between the *freeze-drying* groups with the *freeze-drying + storage* group ( $p < 0.01$ ).

For Visual Score variables there was no significant difference between fresh groups with *freeze-drying* groups ( $p > 0.05$ ), but there are significant differences between fresh groups with *freeze-drying + storage* group ( $p < 0.01$ ) and between *freeze-drying* groups with *freeze-drying + storage* group ( $p < 0.01$ ). For the variable percentage of tail DNA there

is no significant difference between fresh groups with *freeze-drying* groups ( $p > 0, 2005$ ), but there are significant differences between fresh groups with a *freeze-drying* + storage group of ( $p < 0.01$ ) and between *freeze-drying* groups with *freeze-drying* + storage group ( $p < 0.01$ ). More detail can be seen in table 5.

Looking at the results of the analysis to the three variables in the three treatment groups above, there are contradictions in the results of analysis of variables between comet fresh groups percentage with a group of *freeze-drying* percentage. For the comets variable percentage there are significant difference (increase) between the fresh groups with the *freeze-drying* group but for visual scores variable and tail DNA percentage obtained no significant difference (increase) between the two groups.

This may occur because of several factors, first there is indeed an increase in the percentage of sperm with damaged DNA caused by *freeze-drying* process, but the degree of damage is low, so it cannot cause a significant increase in the Visual Scores and Percentage of its tail DNA.

Second, there is actually a low degree of DNA damage in a sample of fresh sperm that not detected by the *Comet Assay*, which can be detected after a *freeze-drying* treatment. Third is the observations subjectivity of comet image on the slide. Nevertheless, the results of a study of comet percentage represents the percentage of sperm with damaged DNA in the two groups is still under research Aitken (2002) in healthy men, i.e. as 85%.

Group of *freeze-drying* + storage shows the significant difference (increase) in terms of percentage of comets, visual scores and percentage of DNA tails compared with fresh group and *freeze-drying* groups, whereas

there was no significant difference (increase) between the groups with the group of *freeze-fresh drying* in the case of visual scores and percentage of DNA tails. Thus, we conclude that *freeze-drying* process itself does not affect sperm DNA integrity, but the process of storage.

Mentioned earlier that the process of *freeze-drying* and storage of sperm should be free of oxygen (Pangestu cited from the Gaylord 2000) and extra-cellular oxidative stress must be minimized to maintain the fertilization potential of spermatozoa (Vishwanath & Shannon 2003).

In this research effort has been made to minimize air leakage by sucking air into the vial *freeze post-drying* with a 5cc syringe inserted through the rubber cap. Air inhaled repeatedly until the syringe piston is pulled back hard. Furthermore, the vial cap is coated with paraffin film before storage at 4° C for 7 days. Micro-organism contamination is also minimal effort using the *freezing* medium, PZ, vials, micropipette and micro centrifuge a sterile tube.

When this study showed significant DNA damage in *freeze-drying* + storage group, possibly this is due to conditions inside the vials during storage was not completely free from the air. Air containing oxygen and water vapor can be a source of reactive oxygen compounds (SOR), it can damage proteins and DNA. Sperm samples exposed to sunlight can cause DNA damage, through a photo excitation process oxygen by ultraviolet light, which changes the *ground state* oxygen ( $O_2^{3\gamma}$ ) into a singlet oxygen ( $O_2^{\gamma}$ ) which is a radical molecules (Sies & Menck 1992). The presence of metal ions as the product of sperm, such as  $Fe^{2+}$  and  $Mg^{2+}$ , which can react with superoxide radicals to form Fenton reaction producing hydroxyl radicals.



Table 5. Summary of Least Significant Difference (BNT) & LSD

Dependent Variables	(I) Group	(J) Group	Mean Difference	SD	Prob
% Comet	Fresh	Freeze Dry	- 11.197	4.152	0.011 ( $\alpha = 0.05$ )
		Freeze Dry +Keep	- 23.624	4.152	0.000 ( $\alpha = 0.05$ )
	Freeze Dy	Freeze Dry +Keep	- 12.428	4.152	0.000 ( $\alpha = 0.05$ )
Visual Score	Fresh	Freeze Dry	- 12.417	13.182	0.353 ( $\alpha = 0.05$ )
		Freeze Dry +Keep	-112.417	13.182	0.000 ( $\alpha = 0.05$ )
	Freeze Dry	Freeze Dry +Keep	-100.000	13.182	0.000 ( $\alpha = 0.05$ )
% Tails DNA	Fresh	Freeze Dry	- 1.814	2.479	0.469 ( $\alpha = 0.05$ )
		Freeze Dry +Keep	- 19.603	2.479	0.000 ( $\alpha = 0.05$ )
	Freeze Dry	Freeze Dry +Keep	- 17.789	2.479	0.000 ( $\alpha = 0.05$ )

## CONCLUSION

*Freeze-drying* does not affect human sperm DNA integrity, but *the freeze-drying* followed by storage at 4° C for 7 days affect sperm DNA integrity. *Comet assay* test showed increased sperm DNA damage in sperm treated with *freeze-drying* followed by storage at a temperature of 4° C during the seven days, compared to fresh sperm and sperm treated with *freeze-drying* only.

Further research on the application *freeze-drying* technique for sperm preservation is still very open, especially about the drying and storage techniques so that the expected effects of ROS (*Reactive Oxygen Species*) can be avoided and the integrity of sperm DNA, where genetic information is stored will be passed on to offspring, can be maintained.

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