Folia Medica Indonesiana Vol. 45 No. 3 July – September 2009 : 185-192

# HUMAN SPERM PRESERVATION USING FREEZE-DRYING
## An Experimental Laboratory Study

**MP Budyandini Dyah Pramesti**
Department of Medical Biology, Faculty of Medicine Airlangga University

## ABSTRACT

*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 . The Posttest Only Control Group Design was used in this study. Population was sperm of adult male over 20 years old. 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 under 4° C storage temperature on human sperm DNA integrity. The posttest-only Control Group Design was Used in this study. 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 sperm should be in normozoopermia category and was obtained with consideration on the method WHO. The sample size was 12, obtained with simple random sampling method. Comet assay was used to determine DNA integrity before freeze-drying, immediately after freeze-drying and 7 days storage after freeze-drying. 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. The results were : no significant difference in DNA integrity between fresh group and freeze-dried group (p>0,05); 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). Manova was used to test the hypothesis. The results Were: no significant difference in DNA integrity Between fresh and freeze-dried group group (p> 0.05), there was very significant difference in DNA integrity Between fresh or freeze-dried group group compared with freeze-dried + 7 days under 4° C storage temperature (p<0.01).*

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

**Correspondence:** MP Budyandini Dyah Pramesti , Department of Biomedic, Faculty of Medicine, Airlangga University, Jl Prof Dr Moestopo 47, Surabaya 60131, Indonesia

## 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 has advantages and disadvantages of each. Kept 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° 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 50-10 years. Potential accidents hit by splashes and spills of liquid nitrogen liquid nitrogen. Floor store room where the tank should be given special protection and nitrogen should be given a specific temperature is -196° 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° C and then dried with a *freeze-dryer.* Sperm is placed in a glass vial, 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° C for 7 days.

Given 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 is relatively simple, rapid and sensitive and can evaluate the integrity of the DNA of single cells (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 ≥21 years, with the results of sperm analysis normozoopermia. The sample is male sperm adults aged ≥21 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 dan 2 b = 1.25 2 a = 1.96 and 2 b = 1.25 ) so that $n = (1.96 + 1.25)^2$ and the result n = 10.3 »12 people

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

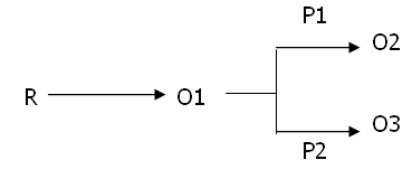


Figure 1. The posttest charge control group design

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° 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 ° C for 7 days

Data analysis methods 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 (AR 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

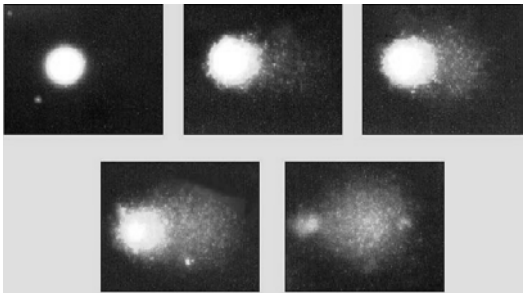

Figure 2. Guidelines for visual scoring of comet images which appears at the fluorescence microscope (AR Collins 2000)

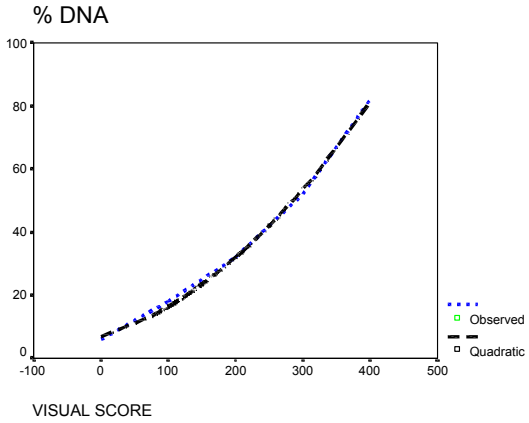


Figure 3.Graph quadratic regression relationship with *Tail%* DNA Visual score (calculated based on the graph Collins 2000)

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 |

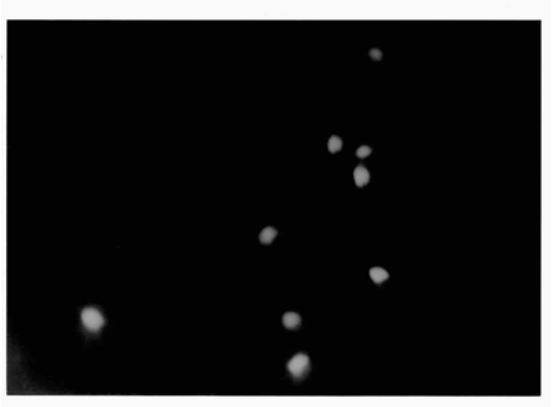

Figure 4 . Image of comet samples of fresh sperm

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 |

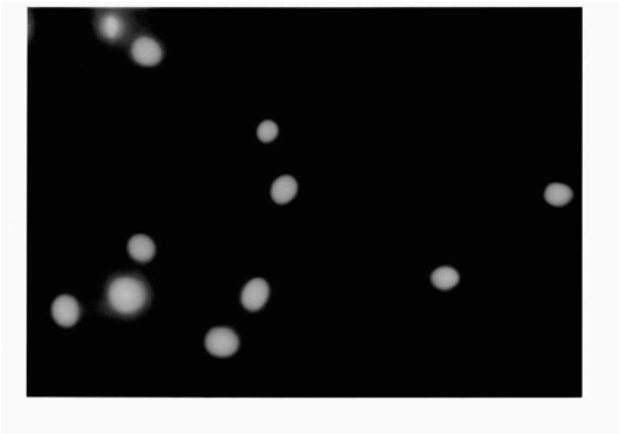

Figure 5 . Image of comet sperm sample post *freeze-drying*

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 |

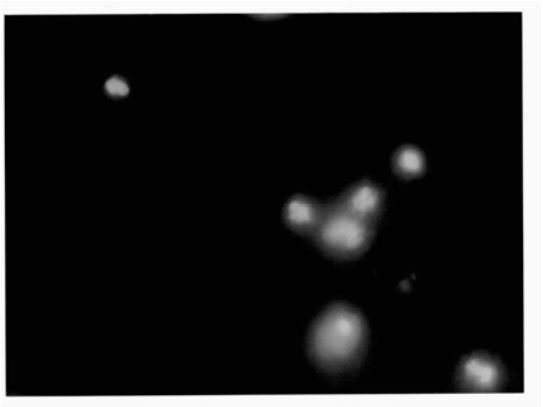

Figure 6. Comet image on sperm samples post *freeze-drying* and storage at 4° C for 7 days

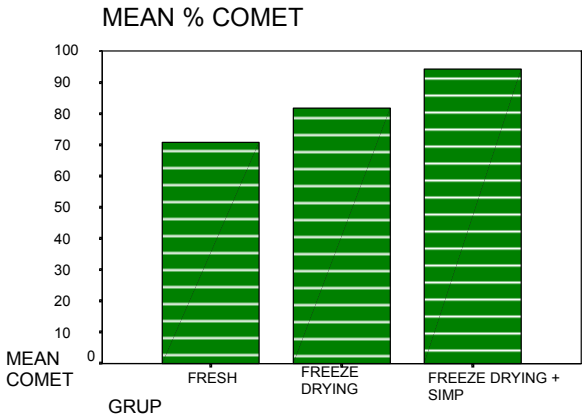


Figure 7. Mean% Comet According to Treatment Group

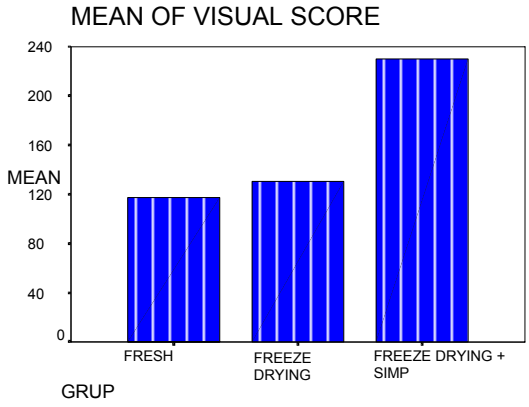


Figure 8. Mean Visual Score According to Treatment Group

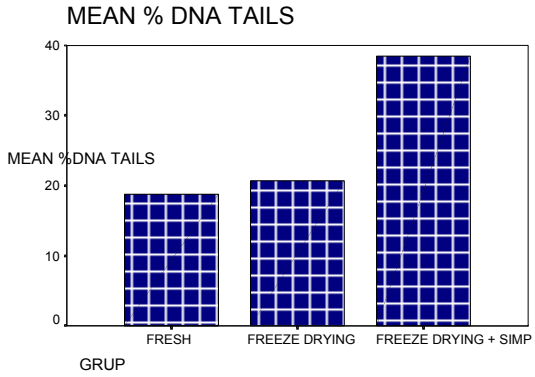


Figure 9.The mean% DNA tails According to Treatment Group

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

Further tests conducted 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 groups with a group of fresh *freeze-drying* + keep ($p<0.01$) and no difference very significantly between the groups with the group *freeze-drying freeze-drying* + keep ($p<0.01$).

For Visual Score variables there was no significant difference between groups with fresh *freeze-drying* groups ($p>0.05$), but there are significant differences between groups fresh with *freeze-drying* + store group ($p<0.01$) and between groups with group *freeze-drying freeze-drying* + keep ($p <0.01$); for the variable percentage of tail DNA there is no significant difference between groups with fresh *freeze-drying* groups ($p>0$, 2005), but there are significant differences between groups with a group of fresh *freeze-drying* + keep ($p<0.01$) and between groups with group *freeze-drying freeze-drying* + keep ($p<0.01$). 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 groups percentage comet fresh with a group *of freeze-drying.* Comets look for the variable percentage difference (increase) was significant between the groups with the group fresh *freeze-drying* but for variable Visual Scores and Percentage of tail DNA obtained no difference (increase) was significant between the two groups.

This may occur because of several factors, among others. Indeed an increase in the percentage of sperm with damaged DNA caused by *freeze-drying* process, but the degree of damage is low, so can not cause a significant increase in the Visual Scores and Percentage of his tail DNA.

Actually there is a low degree of DNA damage in a sample of fresh sperm that are not detected by the *Comet Assay,* which can be detected after a *freeze-drying* treatment. 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 difference (increase) was significant in terms of percentage of comets, Visual Scores and Percentage of DNA tails compared with a group of fresh and *freeze-drying* groups, whereas there was no difference (increase) was significant between the groups with the group *of freeze-fresh drying* in the case of Visual Scores and Percentage of DNA tails. Thus can be concluded 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 in this study showed significant DNA damage in group *freze-drying* + storage, possibly this is due to conditions inside the vials during storage was not completely free from the air. Air containing oxygen and water vapor which can be a source of reactive oxygen compounds (SOR), which 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°]) into a singlet oxygen ($O_2$ [5°]) 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 ($\alpha = 0.05$) |
| % Dna Tails | 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.

## REFERENCES

Aitken RJ. 2002. DNA damage in 85% of human sperm. AFH Library, www.alkalizeforhealth.net/L spermdamage.htm, down loaded at 21/11/2002

Collins AR. 2000. Theoretical and practical aspects of The comet assay. Rowett Research Institute, Aberdeen, UK. E mail : <a.collins@rri.sari.ac.uk>

Gaylord B. 2000. Young inventors award : Gold Award : Biology, An Indonesian PhD student devises a simple, longterm and cheap way to store organic materials. *Far Eastern Economic Review*, December 21st 2000. www.feer.com archive, down loaded at Feb 2nd 2002

Morris ID, Ilott S, Dixon L and Brison DR. 2002. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Human Reproduction*; 17(4) : 990-8

Pangestu M. 2002. Preservation of Spermatozoa : Method and Application. *Reprotech*; 1(2):55-6

Rojas del C E. 2000. The use of The single cell gel electrophoresis assay for human biomonitoring studies

Sies H, Menck CFM. 1992. Singlet Oxygen Induce DNA Damage. *Mutation Research*, 275:367-375

Ward MA, Kaneko T, Kusakabe H, Biggers JD, Whittingham DG, Yanagimachi R. 2003. Long Term Preservation of Mouse Spermatozoa after Freeze-Drying without Cryoprotection. BOR Papers in Press. Published on August 20, 2003 as DOI : 10.1095/biolreprod.103.020529