DEVELOPMENT OF CONDITIONS FOR COMET ASSAY APPLICATION IN FORENSIC INVESTIGATION OF RAPE AND OTHER SEXUAL ASSAULTS

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ABSTRACT

The problem of personal identity in rape cases is solved by DNA analysis as a standard procedure in forensic laboratories. However, fixing of the exact time of the sexual abuse is still an unsolved issue in the forensic science. Here, we present our attempt to apply the method of Comet assay to measure the kinetics of sperm DNA degradation. Modification of the procedure has enabled us to obtain a good correlation between time of ejaculation and the time of test performance. We see this work as a proof of the principle that sperm DNA degradation could be used as a molecular clock for better estimation of the time when rape has occurred. A vast database built from the work of many laboratories is required in order such a phenomenon to be used in practice by forensic scientists.

Keywords: forensic, Comet assay, spermatozoa

Introduction

In recent years the techniques for DNA analysis have improved in terms of sensitivity (4, 5). DNA analysis in the forensic medicine, aiming to determine one's identity has developed with an extremely fast rate. The advances of forensic DNA testing methodologies led to requirement of extremely smaller amounts of genetic material in order to produce a given profile and it is relatively easy to find identity in rape cases through analysis of semen material. However, a persistent problem that has long plagued forensic pathologists has been the determination of the time since the rape. The idea of using the decay of macromolecules in forensic science is not new. DNA is relatively stable molecule. Quite surprisingly, fragments of DNA, suitable for analysis, could be preserved for up to 100 000 years (1, 3). Therefore, DNA may be preserved from degradation at least in some cells for quite long periods of time. It was shown that DNA degradation could be used in forensic science for the assessment of the time of death (2). In sexual assault cases dried semen fluid, which usually can be found on cloth, includes spermatozoa. One of the first steps in the investigative process in rape cases is to identify the presence of spermatozoa. The process of proving the presence of semen liquid in dried findings, following the rules of forensics' practice, is done most often by microscopic search for whole spermatozoids or through looking for distinctive ingredients of the sperm like the presence of the Y chromosome or Prostate Specific Antigen (2). The next step is to carry out DNA profiling on the evidential material e.g. swab.

It is tempting to assume that there is a significant association between DNA degradation and the time since the intercourse. Moreover, in the sperm DNA is compacted more tightly by protamines in the spermatozoid heads. This tight compaction presumably leads to higher resistance of DNA to the action of nucleases and genotoxins. In addition, less enzymatic activities have been found in the head of spermatozoa. Some degradation of DNA in the spermatozoa does exist and has been assessed in several studies. It was shown that degradation could be initiated by stress conditions or by changes in chromatin structure (6, 7). As DNA degradation in spermatozoa unavoidably takes place, it is of interest to make a kinetic analysis of this degradation. Such information could be very useful in the field of forensic science.

In order to better understand the process of sperm DNA degradation along the time we designed our own conditions for Comet assay. By using these conditions we were able to pursue the process not only in the semen but also in dried on a piece of cloth spermatozoa.

Materials and Methods

All reagents were purchased from the Sigma-Aldrich company unless stated otherwise.

Semen collection

Samples from sperm ejaculates of healthy, donors have been collected in sterile tubes. Spermograms were performed on each sample in order to confirm normozoospermia of all individual semen liquids.

Sample preparation and manipulation

100 µl of cell sperm ejaculates were dropped on 3x3 cm clean cotton cloths. The last were stored at room temperature for the time-course studying.

Comet assay on spermatozoa

Liquid aliquots of 20 µl of the same ejaculates were preserved in 1.5 µl eppendorf tubes as control probes for assessing the DNA degradation in semen. Liquid probes were stored at 4°

C until the time of the experiment. Spermatozoa cells were extracted from dried cloths by soaking for 5 min at 37°C and then subsequent washings with 200 µl of PBS buffer. Spermatozoa cells were centrifuged at 3500 rpm/min and next, resuspended in 1 ml of PBS.

The sperm suspesion (approximately 2 -10^5 sperm cells) was mixed with 0.7% agarose and dropped on microscopic slides, precoated with agarose. The slides were transferred to two different solutions for enzyme treatment for specific time periods, crucial steps for decondensing sperm chromatin and allowing migration of broken DNA out of the nucleus. Slides were electrophoresed under neutral conditions at 9 V and 130 mA for 10 minutes at 10°C. After that the slides were air-dried. The comets in the gel were stained with SYBR green I (Molecular Probes) and visualized under fluorescent microscope Leitz (Orthoplan, VARIO ORTHOMAT 2) using 450-490 nm bandpass filter. Pictures were taken with a build-in microscope photo camera.

Results and Discussion

The estimation of the time since rape or other sexual assaults is a problem in the forensic investigation. An intriguing question is whether kinetics of DNA degradation in the spermatozoa could be a "molecular clock" for an exact estimation of the time since the ejaculation. We placed drops (100 µl each) of semen taken from healthy donors on 5 pieces of cotton cloths for each individual. In order to imitate a real situation the pieces of cloths were kept in paper envelops at room temperature without additional precautions for preservation of the spermatozoa. As controls part of the semen fluid was kept in tubes in a fridge at 4° C. On the second, forth, seventh and tenth day after sample collection spermatozoids were washed from cloths in a tube (see Material and Methods) and were subjected to Comet assay.

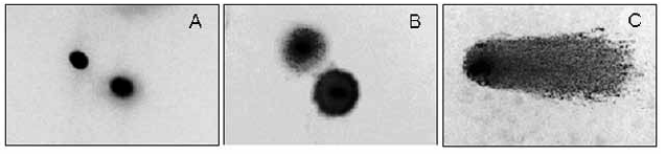

Fig. 1. Comet assay on spermatozoa cells: A – sopermatozoa cells; B – Spermoplast; C – Comet from spermatozoid.

Examples of comets obtained by us from spermatozoa are presented on **Fig. 1**. Wang et al. and Trisini et al. have been studied the degradation of DNA in spermatozoa. These authors, however, did not follow the degradation over a time period (6, 7). It has to be noted that by our modifications of the method we obtained comets from spermatozoa resembling those of somatic cells (**Fig. 1D**). After the enzymatic disruption of the heads of the spermatozoids (**Fig.1 A**) we obtained objects with spread chromatin (**Fig. 1B**). By analogy with spheroplasts (yeast cells without cell wall) we called such objects spermoplasts. In these objects DNA has not been degraded. Spermatozoid with partially degraded DNA (comet) is shown on **Fig. 1C**.

On **Fig. 2** an example of the kinetics of DNA degradation in liquid probes and from dry probes is present. The number of comets from spermatozoa is presented as a percentage from the whole objects counted (spermoplasts and comets). As can be seen from the figure DNA is subjected to degradation both in the liquid and dried probes from the cloths. It has to be noted that, although, for each individual the kinetics of DNA degradation was different, there was always an increase of DNA degradation with time.

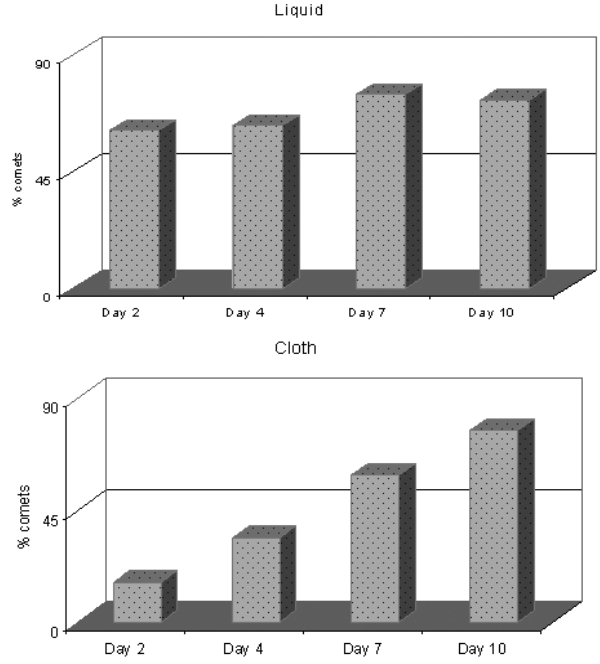

Fig. 2. Kinetics of DNA degradation in spermatozoa cells revealed by Comet assay.

Conclusions

These results, although promising, are preliminary and represent only a proof-of-principle that DNA degradation could be a "molecular clock" in crime investigations. Results from many groups and laboratories obtaining huge database are required in order such a phenomenon to be used in practice by forensic scientists.

REFERENCES

1. **d'Abbadie M., Hofreiter M., Vaisman A., Loakes D., Gasparutto D., Cadet J., Woodgate R., Pa¨a¨bo R., Holliger P.** (2007) Nature Biotechnol., **25**, 939-943.
2. **Johnson L.A., Ferris J.A.J.** (2002) Forensic Sci. Intenation., **126**, 43-47.
3. **Pa¨a¨bo S.** et al. (2004) Annu. Rev. Genet., **38**, 645-679.
4. **Saks M.J, Koehler J.J.** (2005) Science, **309**, 893-895.
5. **Tie J., Uchigasaki S., Oshida S.** (2008) *International Medical J.,* **15**, 307-314.
6. **Trisini A.T., Narendra B.S., Singh P., Duty S.M., Hauser R.H.** (2004) Fertility and Strerility, **82**, 1623-1632.
7. **Wang P., Shao C., Shen D., Li Q., Liu Z., Fu X., Li H.** (2008) The effect of freeze drying on the deoxyribonucleic acid chain of human sperm. 2nd International Conference on Bioinformatics and Biomedical Engineering.