

*Short Communication*

## ***Ex Vivo* Time-Dependent Cell DNA-Degradation Shown by Single Cell Gel Electrophoresis**

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Single cell gel electrophoresis, also known as the comet assay, is a recently developed method for detection of DNA damage in various types of nucleated mammalian cells. The purpose of this study was to investigate the effect of time and storage medium on the *ex vivo* formation of single cell gel electrophoresis-detectable DNA damage in lymphocytes from man and rat, and bone marrow nucleated cells from rat. For this assay, the isolated cells under investigation were embedded in agarose, lysed and treated with alkaline condition to unwind DNA. Breaks in the DNA will allow it to extend into comet-like shape toward the anode during electrophoresis. Thus, the method can detect DNA damage in individual cells (Singh *et al.* 1988; McKelvey-Martin *et al.* 1993). Although the exact basis for the comet formation is obscure, single cell gel electrophoresis has proven useful in measuring chemical and radiation-induced DNA lesions (Olive *et al.* 1993; Vijayalaxmi, Strauss & Tice 1993), aging-induced DNA degradation, resistance level of cells to some agents and the ability of DNA repair. The technique also permits the detection of oxidative damage to pyrimidines of DNA after relevant modifications (Collins *et al.* 1993 & 1995).

Peripheral lymphocytes from Sprague-Dawley rats and one healthy human were isolated with Histopaque 1083 and 1077 (Sigma Chemical Co., St. Louis, MO, U.S.A.), respectively. Bone marrow from one rat femur was flushed out with 3 ml of cold phosphate buffered saline (0.1 M, pH 7.4) supplemented with 10 units/ml heparin, the other femur was flushed with Eagle medium (GibcoBRL, Scotland, U.K.) supplemented with 10 units/ml heparin. Aliquots of cells were separated and suspended in PBS and Eagle medium, respectively, and washed twice with 1 ml of the relevant medium. The cells were then resuspended and diluted to about 500,000 cells per ml. The sample with Eagle medium was then divided into two aliquots, one had foetal calf serum

(GibcoBRL) added to a final concentration of 10%. The complete preparation of cell suspension lasted about 30 min. from the sampling. All suspensions were kept at 4°. Comet assay was carried out after storage of the suspension at 1.5, 3, 4.5, 6, 8, 12, 16 and 20 hr.

*Comet assay.* The technique by Singh *et al.* (1988) was used with minor modifications. Eighty-five  $\mu$ l of 1% normal melting agarose (type I-A, Sigma Chemical Co.) in  $Mg^{++}$  and  $Ca^{++}$  free phosphate-buffered saline (0.1 M, pH 7.4) (GibcoBRL) was dissolved and spread onto the frosted part of a glass slide (Socorex, Swiss) and covered with an 18 $\times$ 18 mm coverslip. Slides were kept at 4° for 10 min. The coverslip was then removed and 10  $\mu$ l of cell suspension was mixed with 85  $\mu$ l of 1% lower melting agarose (type VII, Sigma Chemical Co.) at 37° and pipetted onto the first agarose layer. The gel was covered with a coverslip and stored at 4° for 10 min. The slide was then immersed into a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM  $Na_2EDTA$ , 10 mM Tris; pH 10 with 1% tritonX-100) at 4° for 1 hr. Electrophoresis buffer (1 mM  $Na_2EDTA$  and 300 mM NaOH) was prepared just before use. Slides were placed in the tank, side by side horizontally and buffer was added to a level of 0.25 cm above the slide and the slides were left in the buffer for 30 min. to unwind the DNA. Electrophoresis was carried out at 25 V at room temperature for 25 min. and the electric current was adjusted to 300 mA by the buffer level. The slide was then neutralized 3 times in a solution of 0.4 M Tris buffer (pH 7.5) and stained for 5 min. with 85  $\mu$ l of 8.5  $\mu$ g/ml acridine orange (Sigma Chemical Co.) in distilled water. An epi-fluorescence microscope equipped with an excitation filter of 490 nm from a 100-W mercury lamp and a barrier filter of 530 nm was used, and within 10 min. after staining the DNA comets were measured with a calibrated scale in the ocular at 400 $\times$  magnification. The measurement from 50 randomly selected cells were averaged. Quantification of the DNA damage was calculated as: Comet tail length ( $\mu$ m) = (maximum head-tail length) - (head diameter). The data came from the average of 50 randomly selected cells in one slide. One-way ANOVA

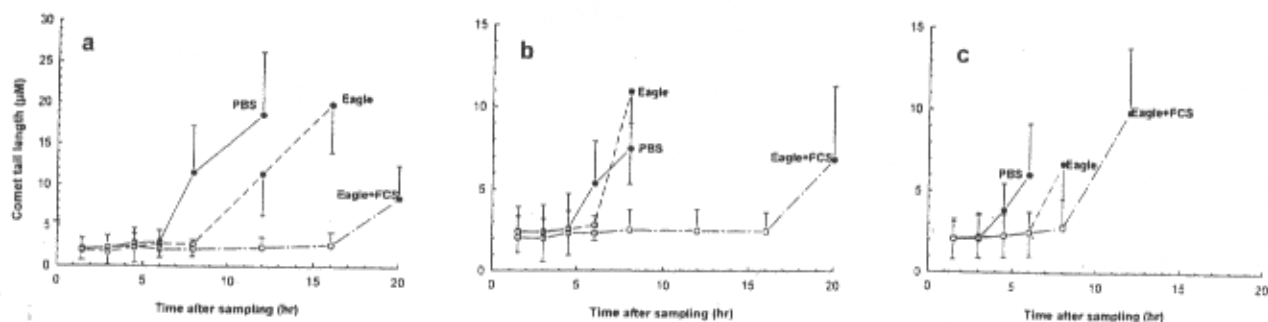


Fig. 1. The influence on comet tail moment of the time period of storage at 4°. a. Human peripheral lymphocytes, b. Rat peripheral lymphocytes, c. Rat bone marrow nucleated cells. PBS=Phosphate buffered saline. Eagle+FCS: Eagle supplemented with foetal calf serum (FCS) to a final concentration of 10%. Closed symbols indicate significantly different from open symbols in post hoc testing at  $P < 0.01$ . Symbols and bars indicate means and S.D.

was used for statistical analysis. Duncan's multiple range test was used for post hoc comparison of means. Differences were considered significant when  $P < 0.05$ .

The major finding of this study is that time after sampling, media condition and origin of the cells all have a considerable influence on the comet assay. The rank order of the time limitation before significant numbers of comets formed spontaneously during storage was rat bone marrow nucleated cells (9 hr), rat peripheral blood lymphocytes (12 hr) and human peripheral blood lymphocytes (16 hr) in phosphate-buffered saline medium; suspension of the cells in Eagle medium delayed the significant increase of the tail length in all cell types and the Eagle medium supplemented with 10% foetal calf serum further delayed the comet formation (fig. 1).

The early background levels of tail length are about 2 µm with only slight variation in the three different cell types: human lymphocytes, rat lymphocytes and rat bone marrow nucleated cells. Storage of less than three hours after sampling regardless of the conditions before starting the comet assay had no influence. Longer storage time had a major impact on the results. Thus we conclude that in order to avoid artefactual DNA breaks, the comet assay should not be delayed after isolation of the cells. However proper choice of medium, i.e. Eagle supplemented with foetal calf serum allows cells to be stored overnight before analysis.

DNA breaks originate from many sources (Eastman & Barry 1992), e.g. direct and indirect attack from exposure to DNA damaging agents, hydroxyl radicals, transient DNA breaks during processes of DNA repair, replication and recombination (Dressler & Potter 1982; Bohr 1990; Wassermann *et al.* 1990), and endonucleases activation during apoptosis (Wyllie *et al.* 1980 & 1981; Duke *et al.* 1983; Barry *et al.* 1990). In addition, reactive oxygen species may be formed by the isolated cells in particular if contaminated with polymorphonuclear granulocytes, and antioxidants depletion may also be a mechanism (Cohen *et al.* 1988; Ward *et al.* 1988). *Ex vivo*, these and other mechanisms may induce artefacts which may be picked up because the comet assay is particularly sensitive. It is therefore very important to have a high degree of standardization in order to avoid

bias by establishing safe handling and storage conditions for each cell type. Since the comet assay has a large potential for experimental genotoxicity and molecular epidemiological studies, the limitations from demands for immediate assay can be overcome by strict standardization.

We conclude that with standardized handling and storage conditions, cells from various sources can be stored for later analysis without artefactual results.

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