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INTRODUCTION OF THE COMET ASSAY FOR THE EVALUATION OF OXIDATIVE STRESS, ANTIOXIDANT EFFECTS AND ENVIRONMENTAL POLLUTANTS EFFECTS ON ANIMAL CELLS ‡

Romana MARINŠEK LOGAR [a)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Zootechnical Dept., Groblje 3, SI-1230 Domžale, Slovenia, Ph.D., M.Sc.

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ABSTRACT

DNA damage within single mammalian cells can be one of the earliest signs of a whole range of health problems, including disease, diet and exposure to occupational or environmental toxins. The Comet assay is a relatively simple and inexpensive method for measuring this damage. It works by determining the number of breaks in the strands of DNA within the cell. Cells are embedded in agarose gel on a microscopic slide and washed to remove the cell membranes, soluble cell contents and histones from the nucleus. An electric field is then activated across and the loops of damaged DNA are pulled towards the anode. Epifluorescence microscopy of stained gels shows the image which gives the techique its name: a clump of undamaged DNA (the head) with the loops pulled away, forming a tail of a comet. We report herein the checking of the Comet assay for the evaluation of oxidative stress on chicken, mouse and pig blood cells, pig sperm cells and chicken hepatocytes. We intend to use the Comet assay as a quantitative method in some nutritional experiments with domestic animals including oxidative food components and antioxidants and for the evaluation of some environmental pollutants effects on animal and human cells.

Key words: molecular genetics / methods / Comet assay / DNA / damage / oxidative stress / antioxidants / environmental pollution

VPELJAVA METODE COMET ZA VREDNOTENJE OKSIDATIVNEGA STRESA, ANTIOKSIDANTNIH UČINKOV IN UČINKOV OKOLIŠKIH POLUTANTOV NA ŽIVALSKE CELICE §

IZVLEČEK

Poškodbe DNK v posamičnih sesalčjih celicah so lahko zgodnji pokazatelji številnih težav pri zdravju, prehrani, izpostavljenosti toksičnim snovem na delovnem mestu in v okolju nasploh. Poskus Comet je relativno enostavna in poceni metoda za merjenje tovrstnih poškodb. Temelji na določanju stopnje prekinitev v verigah celične DNK. Celice vklopimo v agarozni gel na mikroskopskih objektnikih in jih speremo, da odstranimo celične membrane, topno celično vsebino in jedrne histone. Pri elektroforezi fragmenti poškodovane DNK potujejo proti anodi. Pri epifluorescentni mikroskopiji obarvanih preparatov dobimo značilno sliko, ki je tehniki dala ime – skupek nepoškodovane DNK (glava) in rep kometa, ki ga tvorijo fragmenti poškodovane DNK. V članku poročamo o preverjanju metode Comet za vrednotenje oksidativnega stresa na prašičjih, mišjih in kokošjih krvnih celicah, prašičjih spermijih in piščančjih hepatocitah. Metodo

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Comet nameravamo uporabiti za kvantitativno vrednotenje v prehranskih poskusih domačih živali, kjer bomo preverjali oksidativne učinke nekaterih prehranskih snovi in vplive antioksidantov, in za preučevanje vpliva nekaterih onesnaževalcev okolja na živalske in človeške celice.

Ključne besede: molekularna genetika / metode / poskus Comet / DNK / poškodbe / oksidativni stres / antioksidanti / onesnaževanje okolja

INTRODUCTION AND LITERATURE REVIEW

Oxygen metabolism produces reactive species that damage protein, lipid and DNA, and this has been suggested as the main cause of the development of several chronic diseases, including cancer (Riso *et al.*, 1999).

DNA integrity is fundamental for transcription, translation and replication in order to avoid mutations and consequently cell senescence or death. It has been evaluated that the amount of oxidative damage, even under normal physiological conditions, may be quite intensive, with estimates as high as one base modification per 130 000 bases in nuclear DNA (Richter *et al*., 1988). There are numerous types of DNA damage: strand breaks (single and double), sisterchromatide exchange, DNA-DNA and DNA-protein cross-links, and base modifications (Simic *et al*., 1989).

DNA damage has been recognised at the oneset of many diseases, such as cancer, and could be a useful biomarker of the oxidative status and the antioxidant defence system of the organism. In literature several methods are reported for studying DNA damage. They include measurement of the modified bases by use of gas chromatography-mass spectrometry with selected ion monitoring, measurement of single base products such as 8-hydroxydeoxy-guanosine (Richter *et al*., 1988) and assays involving repair endonucleases (Riso *et al.*, 1999).

A relatively simple technique called Single Cell Gel Electrophoresis or COMET assay has been introduced in 1978 by Rydberg and Johanson to evaluate DNA damage (specifically single strand breaks and alkaline–labile sites) in any eucariotic cell population that can be obtained as suspension of single cells (Nelms *et al*., 1997). This method has been mainly applied to quantify DNA damage and DNA repairing capacity following UV, ionising radiation, and oxidative damage in *in vitro* models and for the evaluation of oxidative stress consequently to *in vivo* treatments (Riso *et al.*, 1999). Comet assay is a highly sensitive and rapid method to investigate the response of single cells to DNA damage agents. This technique allows us to detect damage levels as low as 0.1 DNA strand breaks per Daltons (Internet address).

Applications of the COMET assay

Duthie and coworkers (Duthie *et al.*, 1996) adopted a molecular epidemiological approach to the following question: do dietary antioxidants present in fruit and vegetables decrease the free radical attack on DNA and hence protect against mutations that cause cancer. In a survey of men 50-50 years of age the supplementation of the diet for 20 weeks with vitamin C (100 mg day^{-1}), vitamin E (280 mg day^{-1}) and β-carotene (25 mg day^{-1}) resulted in a highly significant decrease in endogenous oxidative base damage in the lymphocyte DNA . In addition the lymphocytes of antioxidant-supplemented subjects showed an increased resistance to oxidative damage when challenged *in vitro* with H_2O_2.

Riso and coworkers (Riso *et al*., 1999) studied the *in vivo* effect of whole tomato consumption on cellular (lymphocyte) antioxidant capacity. The resistence of lymphocytes to oxidative damage when challenged *in vitro* with H_2O_2 resulted higher after the period of tomato puree intake than after the tomato free diet. As a whole food instead of a pure substance was used, it was impossible to conclude which is the main compound involved in the protection of DNA

from oxidative stress. Tomato contains in fact a lot of different substances (vitamins, carotenoids, flavonoids) whose importance in enhancing the protective mechanisms of cells is yet under study. To our knowledge, apart from Duthie *et al*. (1996) and Riso *et al*. (1999) no other paper has reported the use of the Comet assay to study the cell resistence to oxidative stress following an *in vivo* supplementation with antioxidants. Anderson *et al*. (1994) succesfully used this technique to study the effect of several antioxidants on primary lymphocytes from two donors but they supplemented cells *ex vivo*. They found an improvement on the cells resistence to the oxidative stress with H_2O_2 as a consequence of the supplementation with some antioxidants under study.

Nelms and coworkers (Nelms *et al*., 1997) successfully used the Comet assay for measuring apoptosis in individual HL-60 cells. Massive fragmentation of cellular DNA makes apoptotic cells easy to distinguish using the Comet assay, as almost the entire volume of DNA migrates outside the head of the comet and most of the DNA of healthy cells remains in the cell nucleus with very little migration in the electric field outside the cell nucleus. Values for calculated tail moments for apoptotic cells are extremely high due to the large fraction of DNA in the tail and the pronounced distance of migration during electrophoresis. A tail moment of greater than about 110 is indicative of apoptosis. After establishing a tail moment cutoff for apoptosis, apoptotic fractions are easily assessed. The Nelms Comet 2.1 software makes the determination of apoptotic cell fractions very easy. The data obtained by Comet assay by Nelms and coworkers were consistent with the results obtained using Promega apoptosis detection system, Fluorescein.

Comet assay statistical analysis

The comets can be succesfully evaluated by the tail moment, defined as the product of tail length and percentage of the fluorescence intensity in the tail. 50 to 100 cells were measured in one experiment and the distribution of tail moments within one sample evaluated. To consider the individuality of each cell, histograms are prepared for the interpretation of results (Bauer *et al.*, 1998).

It was demonstrated that the distribution of tail moments of individual cells within one sample is not represented by a Gaussian distribution. Therefore the widely used mean value of tail moment is not an ideal parameter to characterise the distribution. The hystograms are thus fitted by the chi-square function which is described by one single parameter, called degree of freedom n. With the curve fit software Sigma Plot (Jandel Scientific, USA) the histograms are superimposed with the chi-square function calculated by the best fit in a non linear regression. The parameter degree of freedom n corresponds to the mean value of tail moments calculated by Gaussian statistics and is therefore a good parameter to describe DNA damage. In order to indicate that the degree of freedom n replaces the mean value of tail moment by Gaussian statistics this parameter is defined as chi-square-mean of tail moments. It has to be considered that the small DNA damage can not be fitted to $n<2$ because the chi-square function has singularity for $n<2$. The curve fitting procedure includes the calculation of standard errors. Each standard error is a measure of the quality of chi-square fit to data points (Bauer *et al.*, 1998).

MATERIAL AND METHODS

Animal cells

We examined whole pig bood and isolated limphocytes, pig sperm cells, chicken whole blood, leucocytes, liver parenchymal cells and mouse whole blood in order to find the most proper testing object for nutritional experiments from the standpoint of the Comet assay as the method for evaluation of oxidative stress and antioxidant effects on domestic animals.

Preparation procedures for animal cells

Nucleated blood cells were isolated (when the whole blood not used) according to the methods described by Johnstone and Thorpe, 1990.
Fresh pig sperm was filtered through 50-mesh nylon, washed in K-Na PBS, pH=7.4 (centrifugation at 600 x g, 10 min, 4^{o} C) and sonicated 20 minutes at maximum power in ultrasonic bath in order to separate sperm heads and tails (Calvin, 1976).
Chicken liver parenchymal cells were prepared by collagenase incubation (Seglen, 1976). Viabilty counts were done by diluting 100 µl of cell suspension with 300 µl of isotonic 0.6 % trypan blue and counting in a Bürker chamber (hemocytometer). Dead and damaged cells are stained blue.

Comet assay procedure (partially modified procedure of Singh *et al.*, 1988)

Rough (unpolished) microscopic slides were used (Curtis Mattesen, UK suppliers, Richardson Supply Co Ltd). 1 % solution of normal melting point agarose (NMP) was brougt by Pasteur pipete on the slide, covered by coverslip, coverslip removed and the agarose gel let to dry at normal room temperature. At the following step 0.6 % NMP agarose was brought on top of the first layer, covered by coverslip and let to solidify in ice plate for 10 minutes. The third layer was a mixture of prepared cells in 0.5 % low melting point agarose (LMP) at 37^{o} C. About 10 000 cells were layered on one slide (the number of cells was determined before by haemocytometer). The cell layer was allowed to solidify for 10 minutes on ice and covered by the last layer of 0.5 % LMP agarose. The coverslip was removed and the slides brought into lysis buffer for at least 1 hour at 4^{o} C (0.03 M NaOH, 1.2 M NaCl, 0.5 % laurylsarcosine, pH>13; for spermatozoa lysing solution contained 1 % Triton X-100, too). The prepared agarose gels were brought into electrophoresys apparatus. Denaturation (40 min) and electrophoresis (20 min) occured in the same buffer (0.03 M NaOH, 2 mM EDTA).The electrophoretic conditions were as following: 25 V and 300 mA. Following electrophoresis the slides were washed three times for five minutes with the neutralisation buffer (0.4 M Tris, pH=7.4). Then the gels were stained with ethidiumbromide (2 µg ml^{-1} of dH_2O) for 20 minutes, rinsed in distilled water and kept in humid environment and dark at 4^{o} C. Olympus epifluorescent microscope at 400 x magnification was used for the examination of slides (100 W Hg lamp, excitation filter of 480 – 550 nm and barrier filter of 590 nm). Photographs were taken by Kodak 400 and Fuji 800 films.

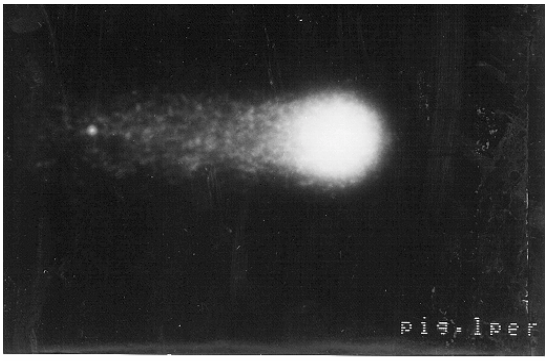
***In vitro* checking of the oxidative stress on animal cells**

In order to check the working of the Comet assay procedure and the quantitative response of the cells on oxidative stress we incubated the experimental cells in different concentrations of H_2O_2 (100 µg, 200µg and 300 µg per liter of PBS) prior to electrophoresis (positive control). Untreated cells were suposed as intact ones.

RESULTS AND DISCUSSION

The Comet assay was originally introduced on human nucleated blood cells and up to our knowledge there were very few Comet assays done on animals. Animal cells pose some special problems, especially chicken blood cells which in contrast with mammalian blood cells have nucleated erytrocytes. Chicken erytrocyte nuclei are very unstable under the conditions of the Comet assay and are the source of a very interfiring backgound at microscopic fluorescence signals examination.

We got good Comet assay results with pig and mouse nucleated blood cells with quantitative response to *in vitro* oxidative stress caused by H_2O_2 treatment: comet tails are longer and more dispersed at higher concentrations of H_2O_2 used (see fig.1 and fig. 3). We did not calculate the variation of the tail moments in terms of H_2O_2 dose, because of preliminary testing and lack of image analysis equipment at the moment of testing. Whole venous blood could be succesfully used for the assay with the minimal risk of high background signal at fluorescence microscopy.



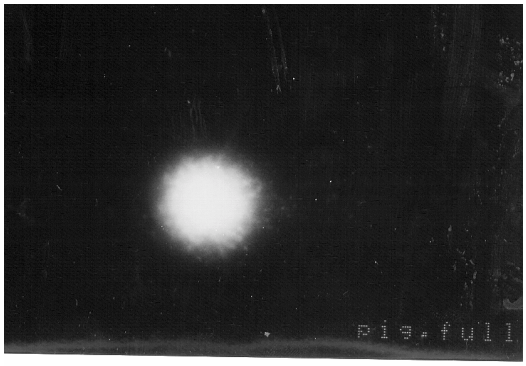
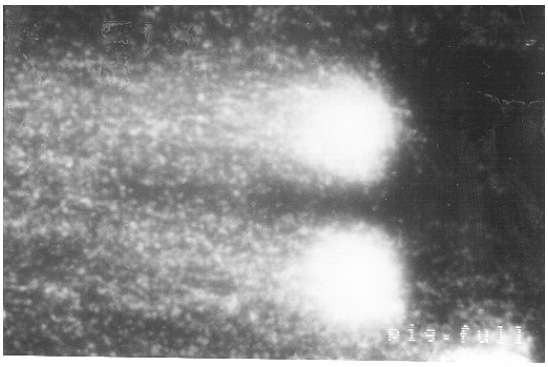


Figure 1 (left). A comet driven from a pig leucocyte nucleus as a result of H_2O_2 (100 μg l^{-1}) treatment and electrophoresis.
Slika 1 (levo). Komet, ki je nastal iz jedra prašičjega levkocita po obdelavi s H_2O_2 (100 μg l^{-1}) in elektroforezi.

Figure 2 (right). Intact pig limphocyte nucleus after electrophoresis.
Slika 2 (desno). Nepoškodovano jedro prašičjega limfocita po elektroforezi.



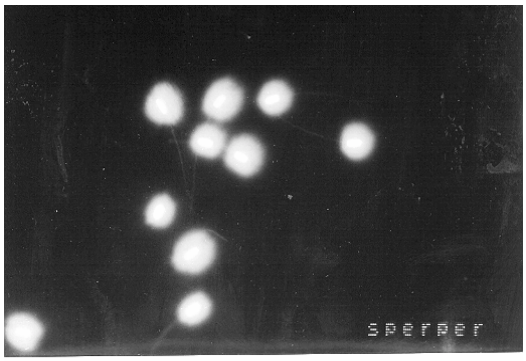


Figure 3 (left). Comets driven from pig leucocyte nuclei as a result of H_2O_2 treatment (300 μg l^{-1}) and electrophoresis.
Slika 3 (levo). Kometi, ki so nastali iz prašičjih levkocitnih jeder po inkubaciji s H_2O_2 (300 μg l^{-1}) in elektroforezi.

Figure 4 (right). No comets were detected following the H_2O_2 treatment (300 μg l^{-1}) and electrophoresis of pig sperm cells.
Slika 4 (desno). Nikakršnih kometov ni bilo zaslediti po obdelavi prašičjih spermijev s H_2O_2 (300 μg l^{-1}) in elektroforezi.

Pig sperm cells that might represent an ideal source of animal cells for the Comet assay did not give positive results. They are beside blood cells the only suspended body cells and this was

the first condition for the Comet assay. Unfortunately (from the view of the Comet assay) they are so resistant to oxidative DNA damage that they did not respond even to the highest concentration of the H_2O_2 used in the assay and thatfore can not be used for Comet assay checking of oxidative stresses. This attribute is consistent with the natural role of sperm cells.

Mouse hepatocytes posed a big resistance to being brought into suspended state. During the collagenase and mechanical treatment of mouse liver over 30 % of cells were damaged as it was indicated by Trypan Blue stainig. Sophysticated methods of liver perfusion would be needed to be more succesful with hepatocyte suspension.

According to the results we decided to use mice in nutrition experiments and check their nucleated blood cells with the Comet assay. Mice would be easy to treat, have good conversion rate and their nucleated blood cells are suitable for the Comet assay.

REFERENCES

Anderson, D./ Yu, T.W./ Phillips, B.J./ Schmezer, P. The effect of various antioxidants and other modifying agents on oxigen-radical-generazed DNA damage in human lymphocytes in the Comet assay. Mutat. Res., (1994), 261-271.

Bauer, E./ Recknagel, R.D./ Fiedler, U./ Wollweber, L./ Bock, C./ Greulich, K.O. The distribution of the tail moment in single cell gel electrophoresis (comet assay) obeys a chi-square not a gaussian distribution. Mut. Res., 398(1998), 101-110.

Calvin, H.I. Isolation and subfractionation of mammalian sperm heads and tails. In: Methods in Cell Biology, Volume XIII, (Ed.: Prescott, D.M.), London, Academic Press, (1976), 85-104.

Duthie, S.J./ Ma, A./ Ross, M.A./ Collins, A.R. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. Cancer Res., 56(1996), 1291-1295.

Fučić, A. Metoda Komete: novi pristup genotoksikološkim istraživanjima. Arh. hig. rada toksikol., 48(1997), 413-419.

Internet address. Http://www.imb-jena.de/~bock/research.html.

Johnstone, A./ Thorpe, R. Immunochemistry in Practice, 2nd Ed., Oxford, Blackwell Scientific publications, (1990), 306 p.

Nelms, E./ Moravec R./ Riss, T. Measuring apoptosis in individual cells with Comet assay. Promega Notes, 64(1997), 13-21.

Riso, P./ Santangelo, A./ Porrini, M. The Comet assay for the evaluation of cell resistance to oxidative stress. Nutr. Res., 19(1999), 325-333.

Richter, C./ Park , J.W./ Ames, B.N. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc. Natl. Acad. Sci USA, 85(1988), 6465-6467.

Seglen, P.O. Preparation of isolated rat liver cells. In: Methods in Cell Biology, Volume XIII, (Ed.: Prescott, D.M.), London, Academic Press, (1976), 30-83.

Simic, M.G./ Bergtold, D.S./ Karam, L.R. Generation of oxy radicals in biosystems. Int. J. Radiat. Biol., 214(1989), 3-12.

Singh, N.P./ McCoy, M.T./ Tice, R.R./ Schneider, E. L. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res., 175(1988), 184-191.