Teratogenesis, Carcinogenesis, and Mutagenesis Supplement 2:43–52 (2003)

Oxygen-Induced DNA Damage in Freshly Isolated Brain Cells Compared With Cultured Astrocytes in the Comet Assay

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Brain cells are continuously exposed to reactive oxygen species generated by oxidative metabolism, and in certain pathological conditions defence mechanisms against oxygen radicals may be weakened and/or overwhelmed. DNA is a potential target for oxidative damage, and genomic damage can contribute to neuropathogenesis. It is important, therefore, to identify tools for the quantitative analysis of DNA damage in models of neurological disorders. The aim of this study was to compare the susceptibility of DNA to oxidative stress in cells freshly dissociated from the mouse brain, to that in cultured brain cells. Both primary cultures and a continuous cell line of astrocytes were considered. All cells were treated by xanthine/xanthine oxidase, a superoxide generator or hydrogen peroxide, applied alone or in the presence of the oxygen radical scavengers, superoxide dismutase, catalase, or ascorbic acid. DNA damage, quantified with the Comet assay, was consistent in all the different cell preparations exposed to oxidative stress, and was attenuated in similar ways by superoxide dismutase and catalase, scavengers of superoxide anion and hydrogen peroxide, respectively. The results with ascorbic acid were more variable, presumably because this compound may switch from anti- to pro-oxidant status depending on its concentration and other experimental conditions. Overall, similar responses were found in freshly dissociated and cultured brain cells. These results suggest that the Comet assay can be directly applied to cells freshly dissociated from the brain of rodents, including models of neurological disorders, such as stroke models and animals with targeted mutations that mimic human diseases. *Teratogenesis Carcinog. Mutagen. Suppl. 2:43–52, 2003.* © 2003 Wiley-Liss, Inc.

Key words: DNA damage; Comet assay; astrocytes; cultured brain cells; stroke; preconditioning; Alzheimer's disease; mice; reactive oxygen species

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Accepted 30 June 2003

Published online in Wiley InterScience (www.interscience.wiley.com) 10.1002/tcm.10079

INTRODUCTION

The brain has the highest metabolic rate of all organs, and depends primarily on oxidative metabolism as an energy source. Oxidative metabolism generates reactive oxygen species (ROS) that can damage a variety of cellular components, including proteins, lipids, and nucleic acids. Endogenous mechanisms for DNA repair are normally very effective in removing spontaneous genetic abnormalities, but increased oxidative stress, or altered defence processes against ROS, in some neurological disorders may overwhelm functional DNA repair and lead to genomic damage in brain tissue. Such a situation occurs with acute injury to the brain in the case of stroke and traumatic brain injury [1–3]. It is also suspected to occur with progressive neurodegeneration, whether age-related (Alzheimer's and Parkinson's diseases) or subsequent to genetic anomalies (e.g., amyotrophic lateral sclerosis, ALS) [4].

Dementias such as Alzheimer's Disease (AD) are also more common in patients who have suffered prior ischemic episodes [5], and oxidative stress may be critical to AD predisposition after ischaemia according to the data outlined next. Fibrillar deposits consisting of amyloid β peptides (AβPs) are a hallmark of AD, and cleavage products of the amyloid precursor protein (APP) [6,7]. Expression of APP is increased following cerebral ischemia [8,9], and AβP production effectively increased after ischemia [10]. The neurotoxic effects of AβPs involve oxidative stress, and disruption of Ca^{2+} homeostasis by ROS [6,7]. Recent data support the latter possibility, since both Ca^{2+} homeostasis and neurosecretion were altered in cells exposed to hypoxic conditions, and at least some of these effects appeared mediated by AβPs [11,12]. Furthermore, suppression of these hypoxic effects by antioxidants indicated the involvement of ROS [13].

In contrast to neurological disorders in which DNA damage may contribute to cell injury and death, an increased tolerance of the brain to insults can be induced by preconditioning (also known as adaptative cytoprotection), the inherent capability of most living cells to enhance their tolerance to cytotoxic stress, and this adaptative capability is due, at least in part, to a higher expression of DNA repair proteins [14]. Recently, we have found that 6 h after preconditioning, the expression of hepatoma-derived growth factor (Hdgf) gene was significantly reduced in the cerebral cortex of mice [15], and low expression of this gene is a feature of established human oesophagal cancer cell lines that are resistant to X-rays [16].

Therefore, it is important to identify and validate a method for quantitative, overall assessment of DNA damage and DNA repair potential in freshly isolated brain cells, in samples collected from the brain of animal models. The single-cell gel electrophoresis (SCGE) or Comet assay is a simple method, through which DNA fragments from single cells are separated by gel electrophoresis, and their subsequent distribution between the body and tail of the comet-shape migration pattern, taken as quantitative index of DNA damage [17,18]. The comet assay is generally used with cultured brain cells [19], but culturing cells harvested from a brain previously exposed to a specific procedure may allow the changes under study to revert, or cells made vulnerable by the procedure to die by apoptosis or necrosis. In addition, basic housekeeping genes may be altered in cultured cells [20]. One way to circumvent these potential limitations is to use the Comet assay on freshly isolated cells.

The aim of this present study, therefore, was to compare, using the Comet assay, the response to oxygen radical damage and radical scavengers of cells isolated directly from fresh brain, dissected rapidly from humanely killed wild-type mice, with cultured brain cells.

MATERIALS AND METHODS

Preparation of Dissociated Cortical Cells From Adult Mice

Adult male C57BL/6 mice (weight 25–30 g; Harlan UK, Blackthorn, UK) were used, with food and water available ad libitum. All animal procedures complied with the British Home Office Animals (Scientific Procedures) Act 1986. Mice were deeply anaesthetized with halothane (5% in O_2:N_2O 1:2) before decapitation. The brain was then immediately removed, and the cortex dissected from subcortical structures in ice-cold phosphate-buffered saline (PBS, without calcium and magnesium) including 200 μM N-1-butyl-α-phenynitrone (PBN; Sigma Chemicals, Poole, UK). Cortical cells were isolated according to Singh [21] with some modifications. The cortical tissue was washed three times with ice-cold PBS to remove most of the red blood cells. After washing, the tissue was placed on the 0.5-mm wire screen of a Tissue Press™ (BioSpec Products, Inc.; Bartleville, OK), then the screen incorporated within the device, and the tissue squeezed through the wire screen while the lower part of the Tissue Press™ was kept immersed in 20 ml ice-cold PBS to minimize the contact of the tissue with air. The tissue suspension was then centrifuged (2,000 rpm for 2 min) and the resulting pellet resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with foetal calf serum (BRL Life Technologies, Inc.; Gaithersburg, MD) and finally dissociated to single cells by gentle triturating through a 5-ml pipette tip and 25- and 27-gauge hypodermic needles, successively. The final volume of cell suspension for a single mouse cortex was 2.5–3 ml.

Cultured Astrocytes

To obtain astrocytes, cerebral cortices were removed from 6–8-day-old Wistar rat pups and placed immediately in an ice-cold buffer solution consisting of 10 mM NaH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 14 mM glucose, 1.5 mM $MgSO_4$, and 3 mg/ml bovine serum albumin. Following the removal of the meninges using fine forceps, the whole cortices were minced gently with a mechanical tissue chopper (McIlwain, MO), and then dispersed into the same buffer containing 0.25 μg/ml trypsin, at 37°C for 15 min. Trypsin digestion was halted by the addition of an equal volume of buffer supplemented with 16 μg/ml soy bean trypsin inhibitor (SBTI, type I-S; Sigma), 0.5 μg/ml DNAase I (type II, bovine pancreas; 125 kU/ml; Sigma Chemicals), and 1.5 mM $MgSO_4$. The tissue was then pelleted by centrifugation at 1,300 rpm for 90 s following which the supernatant was removed and the cell pellet resuspended in 2 ml of buffer solution containing 100 μg/ml SBTI, 0.5 μg/ml DNAase I, and 1.5 mM $MgSO_4$. The tissue was subsequently triturated gently with a fire polished Pasteur pipette. After allowing larger pieces of tissue to settle for 5 min, the cell suspension was taken and centrifuged at 1,300 rpm for 90 s before resuspension into 60 ml of culture medium (Eagle's minimal essential medium supplemented with 10% FCS and 1% penicillin-streptomycin (GIBCO, Paisley, Scotland). The cell suspension was then aliquoted into 2 $\times$ 25 cm^2 flasks and onto glass cover slips in 6- and 24-well

tissue culture plates. Cells were then kept in a humidified incubator at 37°C (95% air; 5% CO_2). This was designated passage 1 and cells were used up to a passage of 2. Four to six hours following plating, cells were washed vigorously several times with fresh media to remove non-adhered cells. This resulted in a culture of primarily type I cortical astrocytes, as confirmed by positive immunostaining with an anti-GFAP antibody. Culture medium was exchanged every 3–4 days and cells were grown in culture for up to 14 days. The astrocytoma cell line, D384, was maintained as previously described [22].

Comet Assay

The chemicals for the Comet assay were purchased from the following suppliers: Roswell Park Memorial Institute (RPMI) 1640 medium from BRL Life Technologies, Inc. (Gaithersburg, MD); agarose, low melting agarose, dimethyl sulfoxide (DMSO), ethidium bromide and trypan blue from Sigma Chemical Company (St Louis, MO); sodium chloride, sodium hydroxide and EDTA from BDH Laboratory Supplies (Poole, UK); phosphate buffer saline (PBS) tablets from Oxoid, Ltd. (Basingstoke, UK).

The cells' viability was checked by trypan blue exclusion [23]. The preparation of the slides, electrophoresis, staining, and slide scoring were as described previously using the modified method of Singh et al. [17, 21], and is described below.

Cell Treatment

Single cell suspensions were treated with each chemical combination under study for 30 min at 37°C in serum-free RPMI 1640. The concentrations of chemicals used were as shown in Results. Cells were treated with hydrogen peroxide or with xanthine/xanthine oxidase as an oxygen radical generating system. The antioxidants/oxygen radical scavengers used were those occurring endogeneously in the body such as catalase, superoxide dismutase, and Vitamin C.

Slide Preparation

The basic alkaline technique of Singh et al. [17] as described by Anderson et al. [24] was followed. Conventional microscope slides were dipped into a solution of 0.05% normal melting agarose (NMA) at about 50°C in Ca^{2+}- and Mg^{2+}-free PBS and allowed to dry on a flat surface at room temperature. This layer was used to promote the attachment of the second and third layers. Around 10,000 treated or control cells were mixed with 75 μl of 0.5% LMA to form a cell suspension. After gently removing the coverslip, the cell suspension was rapidly pipetted onto the first agarose layer and spread using a coverslip, and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the coverslip, the third layer of 0.5% LMA (75 μl) at 37°C was added, spread using a coverslip, and again allowed to solidify on ice for 5 min. After removal of the coverslip, the slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM Na_2 EDTA, and 10 mM Tris, pH 10), with 1% (v/v) Triton X-100 and 10% DMSO added just before use for a minimum of 1 h at 4°C.

Electrophoresis

The slides were removed from the lysing solution, drained, and placed in a horizontal gel electrophoresis tank side by side, avoiding spaces and with the agarose ends facing each other, nearest to the anode. The tank was filled with fresh electrophoresis solution (1 mM Na_2 EDTA, and 300 mM NaOH) to a level approximately 0.25 cm above the slides. The slides were left in the solution for 30 min to allow the unwinding of the DNA and expression of alkali-labile damage before electrophoresis. Electrophoresis was conducted at 4°C for 30 min, using 25 V and adjusting the current to 300 mA by raising or lowering the buffer level and using a compact power supply (2300 Microdrive 1, Pharmacia-LKB, Uppsala Sweden). All of these steps were conducted under dimmed light (apparatus covered with a black cloth) to prevent the occurrence of additional DNA damage. After electrophoresis, the slides were placed horizontally, Tris buffer (0.4 M Tris, pH 7.5) was added dropwise and gently to neutralise the excess alkali, and the slides were allowed to sit for 5 min. This neutralising procedure was repeated three times. Slides were then dried in methanol for 5 min, and stored in a low humidity environment before staining.

Staining

The slides were rehydrated in distilled water. Eighty-five microliters EtBr (20 μg/ml) was added to each slide and covered with a coverslip. The slides were placed in a humidified air-tight container to prevent drying of the gel and immediately analysed.

Slide Scoring

Slides were examined at $\times 625$ magnification resulting from a $\times 40$ objective, a 12.5 projection lens and a 12.5 eyepiece on a fluorescence microscope (Leica, Weztler, Germany), equipped with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. A CCD camera was attached to the microscope and the images of cells displayed on the associated computer monitor screen at a magnification of $\times 1{,}000$. Twenty-five cells were scored from each replicate slide (i.e., 50 cells in total).

Computerized Analysis

A computerized image analysis system (Comet 4.0, Kinetic Imaging, Ltd, Liverpool, UK) was used to measure Comet parameters. The parameter selected for presentation was the tail moment. The tail moment, equivalent to the integrated value of density multiplied by migration distance, is considered to be the most sensitive measurement, and it was automatically generated by the computer. The system setup for this equipment was as follows: head threshold, 2%; tail threshold, 0%; smoothing value, 1; background height, 20; and tail break length, 5.

Statistical Analysis

There are several ways of processing Comet assay data [25]. The tail moment data violated the assumption of normality and the equal variance test

(homoscedasticity) required for the parametric analysis of variance (ANOVA) procedure. However, logarithmic transformation of the tail moments can often introduce normality prior to statistical analysis. Alternative non-parametric approaches are available using Mann-Whitney or Kruskal-Wallis test procedures, as appropriate. Normality or log-normality was validated using the Kolmogorov-Smirnov Test. In addition, intra-sample comparisons of the replicates were conducted using the Mann-Whitney test to verify that the results were consistent, as a necessary prerequisite to pooling samples.

RESULTS

The responses of freshly isolated brain cells, primary astrocytes, and a continuous cell line of astrocytes are shown in Figure 1. Xanthine and xanthine oxidase alone, as well as the two different concentrations of xanthine and xanthine oxidase in combination, increased DNA damage. Catalase diminished these effects, SOD also (albeit only slightly), but vitamin C did not. In fact, vitamin C exacerbated DNA damage in freshly isolated brain cells.

A similar pattern of responses was obtained with hydrogen peroxide combined with catalase, SOD, and vitamin C (Fig. 2).

The dose-response relationships in the cultured astrocytes (continuous cell line) with xanthine/xanthine oxidase, hydrogen peroxide, and the radical scavengers

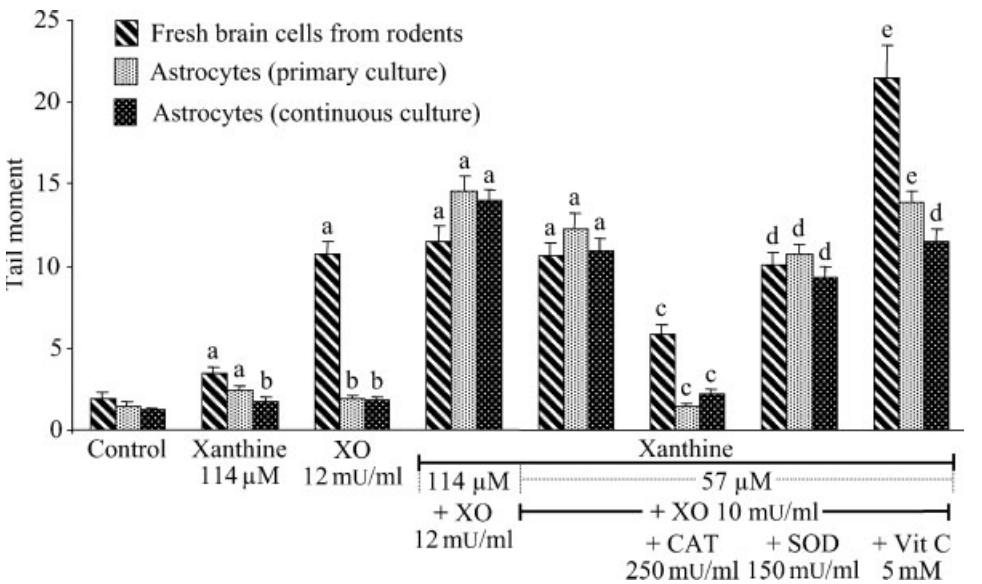

Fig. 1. DNA damage, assessed in three different types of brain cells, in controls and subsequently to their exposure to oxidative stress induced by xanthine/xanthine oxidase, with or without the presence of protective agents. DNA damage was assessed by the Comet assay and expressed as tail moment. Statistical analysis: [a]Significant increase ($P<0.001$), comparison with the negative control; [b]no significant difference, comparison with the negative control; [c]Significant decrease ($P<0.001$), comparison with xanthine/xanthine oxidase; [d]no significant difference, comparison with xanthine/xanthine oxidase; [e]significantly increased ($P<0.001$), comparison with xanthine/xanthine oxidase.

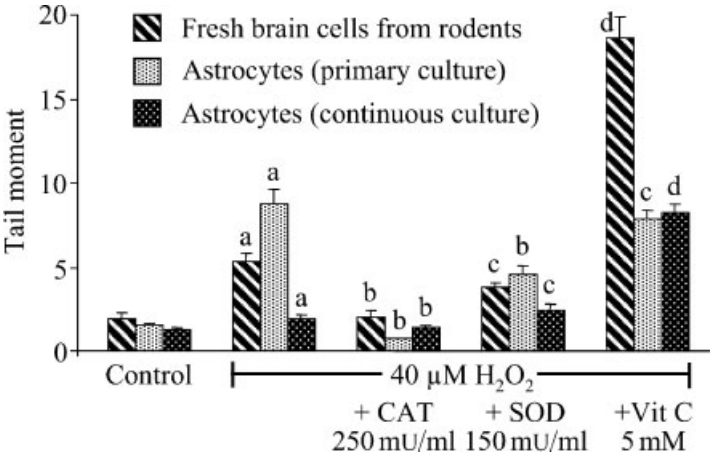

Fig. 2. DNA damage, assessed in three different types of brain cells, in controls and subsequently to their exposure to oxidative stress induced by hydrogen peroxide, with or without the presence of protective agents. DNA damage was assessed by the Comet assay and expressed as tail moment. [a]Significant increase ($P<0.001$), comparison with the negative control; [b]Significant decrease ($P<0.001$), comparison with hydrogen peroxide; [c]no significant difference, comparison with hydrogen peroxide. [d]significantly increased ($P<0.001$), comparison with hydrogen peroxide.

investigated are shown in Figure 3. With xanthine/xanthine oxidase and hydrogen peroxide there was a marked reduction in response with catalase, but lesser effects with SOD and vitamin C.

DISCUSSION

Cells tended to respond in a similar way to catalase and SOD after treatment with xanthine/xanthine oxidase and hydrogen peroxide, even when dose response relationships were examined. The response to ascorbic acid was less predictable, and this could have been anticipated, depending on the dose and the cell system used, as ascorbic acid can act as a pro- or antioxidant manner [e.g., 24].

Thus, it would appear that freshly isolated brain cells produce similar responses for oxygen radical generated damage as cultured astrocytes (whether from primary or continuous cultures). This would suggest that all these cells may be used to study mechanisms of DNA repair, in animal models of neurological disorders and their corresponding control (e.g., sham-operated animals). Indeed, it has been shown already that 4 hours after occlusion of the middle cerebral artery (MCAO; i.e., a classical stroke model) in rats, the percentage of DNA damage in the Comet assay increased 3- to 4-fold in the brain regions that are made selectively ischaemic with this procedure [26].

We are currently investigating oxidative repair in cells isolated from preconditioned and sham-treated mouse brain cells, with a view to a better understanding of the preconditioning process. Preconditioning refers to the adaptive cytoprotection that can be induced by a variety of sublethal insults such as a short period of hypoxia, which increases the brain resistance to a subsequent, potentially

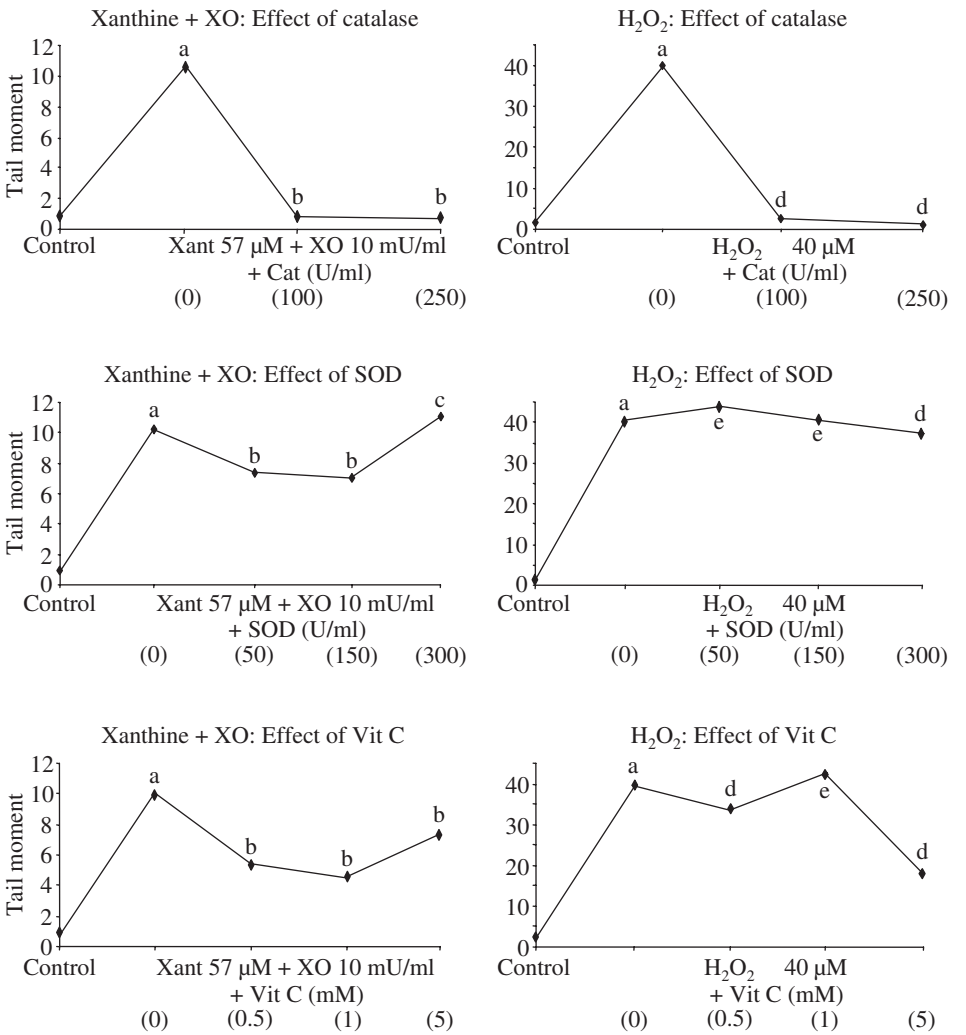

Fig. 3. Concentration-dependent effects of the radical scavengers catalase (Cat), superoxide dismutase (SOD), and vitamin C (Vit C) on DNA damage produced by xanthine/xanthine oxidase and hydrogen peroxide (**left** and **right**, respectively) in cultured astrocytes (continuous cell line). DNA damage was assessed by the Comet assay and expressed as tail moment. [a]Significant increase ($P<0.001$), comparison with the corresponding negative control; [b]significantly decreased ($P<0.001$), comparison with xanthine/xanthine oxidase; [c]no significant difference, comparison with xanthine/xanthine oxidase; [d]significantly decreased ($P<0.001$), comparison with hydrogen peroxide; [e]no significant difference, comparison with hydrogen peroxide.

lethal insult, e.g., severe ischemia. Dementias such as Alzheimer's Disease (AD) are also more common in patients who have suffered prior ischemic episodes [5], and oxidative stress could also be critical to AD predisposition after ischaemia. It has become relevant also and timely to examine DNA damage/repair by applying the Comet assay to cells prepared from transgenic mice, since a variety of animals with targeted mutations mimicking human neurodegenerative disorders are already

available [27]. The present study suggests strongly that the Comet assay is a useful and sensitive technique for the detection of such responses in a wide variety of models, both in vitro and in vivo.

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