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# The Semi-quantitative Comparison of Oxidative Stress Mediated DNA Single and Double Strand Breaks using Terminal Deoxynucleotidyl Transferase Mediated End Labeling Combined with a Slot Blot Technique

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The accumulation of DNA damages by environmental stresses is represented by the steady state level of single strand breaks (SSBs) and double strand breaks (DSBs). Terminal deoxynucleotidyl transferase (TdT) mediated end labeling is suitable in detecting DSBs, but is unsuitable for SSBs due to its catalyzing characteristics. However, the sensitivity of TdT to detect SSBs may be significantly improved by first denaturing the double strands and expose all the DNA nicks as potential substrates for TdT. By coupling DNA denaturation to slot blot southern hybridization, the authors demonstrate the sensitive detection of SSBs as well as DSBs in 20 ng DNA samples derived from a retinal pigment epithelial cell line treated with tert-butyl hydroperoxide. The signal intensity of denatured and TdT-treated DNA in slot blot hybridization correlated to the amount of SSBs calculated in an S1 nuclease digestion assay. The signal ratio between denatured and non-denatured DNA likely approximates the SSBs/DSBs ratio in genomic DNA. The combination of DNA denaturing, TdT treatment and slot blot hybridization could be a useful method to assess oxidative stress-induced DNA strand damages.

*Keywords*: DNA strand break; Terminal deoxynucleotidyl transferase; *tert*-Butyl hydroperoxide; Slot blot

## INTRODUCTION

Genomic DNA damage induced by oxidative stress initiates several biological cellular dysfunctions such as apoptosis or senescence.[1–4] Single strand breaks (SSBs) or double strand breaks (DSBs) in genomic DNA by oxidative stress usually result in immediate cellular repair,[5–9] thus the steady state level of SSBs and DSBs is a good indicator of cell stresses. DNA polymerase-1 nick translation labeling and terminal deoxynucleotidyl transferase (TdT) labeling assays are enzymatic labeling techniques used to detect DNA strand breaks.[10,11] Both methods detect single and double strand breaks in DNA, although the former is predominantly used in SSB labeling and the later for detecting DSBs due to their catalyzing characteristics.[12,13] Although the sensitivity of TdT to detect SSBs is thought to be limited,[12,13] an undertaking to improve the sensitivity of the assay for SSBs detection may enable the semi-quantitative comparison of SSBs to DSBs using the same enzyme. We have investigated the sensitive detection of oxidative stress mediated SSBs using TdT mediated nick end labeling combined with slot blot hybridization.

## MATERIALS AND METHODS

### Cell Culture

The ARPE-19 cell line was maintained in Dulbecco's modified Eagle's medium/Nutrient mixture F12 with 15 mM Hepes buffer (DMEM/F12; BioWhittaker, Walkersville, MD) + 10% fetal bovine

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serum (FBS; UBI Upstate, Lake Placid, NY), 0.348% additional sodium bicarbonate, and 2 mM L-glutamine solution (GIBCO, Grand Island, NY) at 20% oxygen conditioned in 10% $CO_2$ at 37°C. For experiments, cells were grown in 75 $cm^2$ flasks at an initial seeding density of 10,000 cells/$cm^2$.

### DNA Extraction and the Induction of Strand Breaks

Genomic DNA was extracted using the Qiagen blood and cell culture DNA mini kit (Qiagen, Santa Clarita, CA). To generate DSBs, DNA samples were fragmented by sonication. The samples were electrophoresed on 1% agarose gels for 2 h at 60 V followed by ethidium bromide (EtBr) staining. For generating SSBs, cells were treated with several concentrations of *tert*-butyl hydroperoxide (tBH) in Hank's balanced salt solution (GIBCO) for 30 min at 37°C prior to DNA extraction. The range of tBH concentration was determined not to affect the viability of ARPE-19 cell line.[14]

### Terminal Deoxynucleotidyl Transferase (TdT) Assay

Twenty or 200 ng DNA samples were treated with TdT (TOYOBO, Osaka, Japan) in the presence of 20μM dATP (Invitrogen, Carlsbad, CA) to generate DNA end poly-A tails. Incubation was carried out at 37°C for 30 min and the reaction was terminated by denaturing the enzyme at 93°C for 5 min. To expose all gaps and nicks as a substrate for TdT, DNA samples were denatured at 95°C for 10 min before the TdT assay.[15,16]

### Slot Blot Analysis

Samples treated with TdT were applied to a Bio-Dot SF Microrofiltration Apparatus (Bio-Rad, Herculus, CA) using alkaline conditions, followed by southern blot analysis with an alkaline phosphatase-labeled oligo-dT probe (24mer) according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, England) and exposed to autoradiographic film as previously described.[17] Autoradiograms were scanned by densitometry and signal intensities were calculated using ImageQuaNT software (Amersham). The sample volume was normalized by quantifying the hybridization signal to a telomeric sequence that is unaffected by DNA damage.[18] Slot blots were stripped by boiling in a 0.1%SDS solution and re-probed with telomeric specific probe $(TTAGGG)_4$.

### S1 Nuclease Assay

The accumulation of SSBs in DNA results in a progressive expansion of DNA smears to shorter molecules with increasing S1 nuclease (Invitrogen) concentration. After treatment with tBH, 1 μg of DNA was incubated with S1 nuclease (1 U/μg DNA) for 30 min at 37°C. The reaction was terminated with 25 mM EDTA. Samples were electrophoresed, stained with EtBr, photographed and the mean DNA fragment length was calculated using an equation previously described,[17] and the number of S1 nuclease sensitive sites was calculated.

### Statistical Analysis

The data were analyzed using the unpaired Student's *t*-test. $p < 0.05$ was determined as statistically significant.

## RESULTS

### DNA Fragmentation and the Signal Intensity of Slot Blot

A dense band of genomic DNA from 1 μg of sample was present at an approximate size of 20.6 kbp. Genomic DNA sonication resulted in a progressive expansion of DNA smears to shorter molecular sizes with increasing sonication times (Fig. 1A). The mean fragment size of DNA sonicated for 0.5, 1 and 2 s was calculated as 14.7, 11.9 and 9.8 kbp, respectively. Signal intensities of 200 ng samples treated with TdT and hybridized with oligo-dT probe were also increased with longer sonication times (Fig. 1B). Samples lacking TdT treatment probed by oligo-dT had quite faint signals that probably represented residual mRNA labeling (Fig. 1B). Signals were increased when DNA samples were denatured prior to TdT treatment. Telomere signals tended to decrease with increasing sonication time; however, the changes were not statistically significant ($p = 0.19$ between control and sonication time 2 s, $n = 3$)(Fig. 1B). The mean fragment size of sonicated DNA based on gel electrophoresis correlated with the signal intensity of the same non-denatured DNA sample on slot blots (Fig. 1C).

### Elution of S1 Nuclease Sensitive Sites in the Samples Treated with tBH

Sizes of genomic DNAs treated with serial concentrations of tBH were identical to untreated sample on agarose gel electrophoresis followed by EtBr staining (Fig. 2A). One unit per μg DNA of S1 nuclease treatment resulted in the progressive increase of DNA smearing to lower molecular sizes with increasing concentrations of tBH (Fig. 2B).

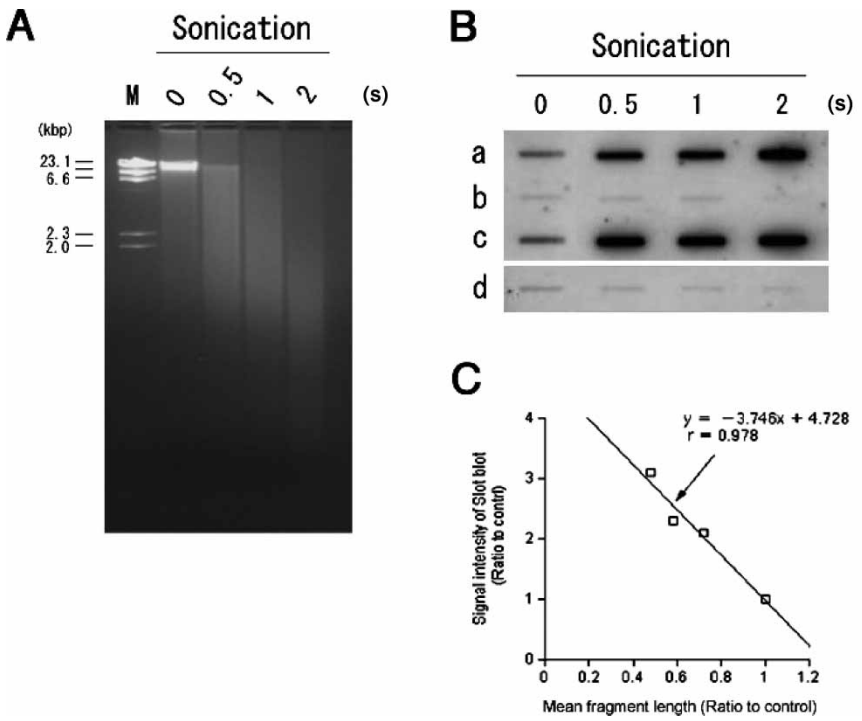


FIGURE 1 (A) Genomic DNA subjected to 1% agarose gel electrophoresis and EtBr staining. (B) Slot blot analysis of fragmented DNA. (a–c) Signals of DNA hybridized with oligo-dT probe. (a) 200 ng of DNA, TdT(+), (b) 200 ng of DNA, TdT(−), (c) 200 ng of denatured DNA, TdT(+) and (d) 200 ng of DNA, TdT(+), hybridized with telomere probe. (C) Data plots for comparing the signal intensity of poly-A labeled DNA (ratio to control) to the calculated DNA fragment size (ratio to control) based on gel electrophoresis analysis.



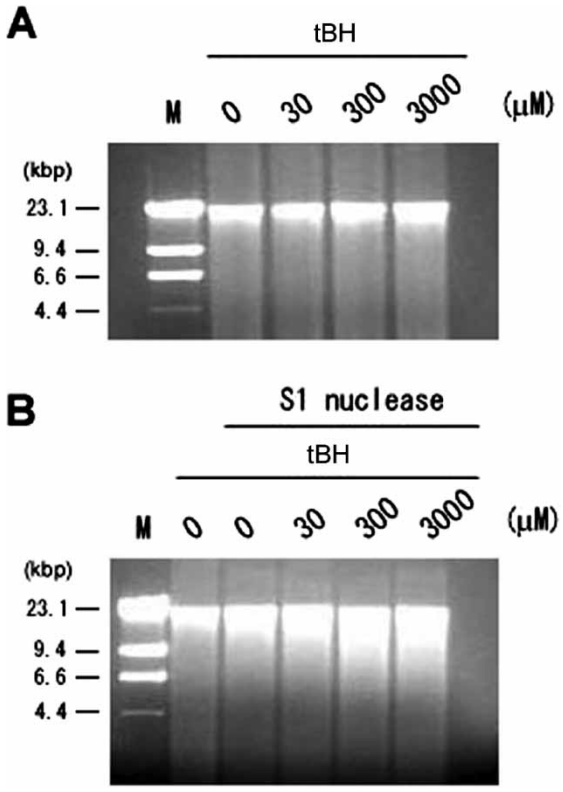


FIGURE 2 One percent agarose gel electrophoresis and EtBr staining after S1 nuclease assay. (A) 1 μg of genomic DNA samples from ARPE-19 cells treated with tBH. (B) Same DNA samples digested with 1 U/μg DNA of S1 nuclease.

### Signals of Poly-A Labeled DNA Strand Breaks in the Samples Treated with tBH

Telomere sequence signal intensities were comparable in the 200 ng samples treated with either tBH concentration (Fig. 3A,C) and control sample. The signal intensities of poly-A labeled, non-denatured DNA from 20 ng samples were mildly increased with tBH concentrations up to 3 mM. The change was statistically significant at 3 mM tBH compared to the control. Signal intensities of samples probed by oligo-dT were remarkably increased by DNA denaturation preceding TdT treatment (Fig. 3A,C). The ratio of signal intensities of tBH treated samples to controls for DNA denaturing, TdT assay, and slot blot methods were highly correlated to the amount of S1 sensitive sites detected in an S1 nuclease assay (Fig. 3B). A significant increase in DNA strand breaks was detected in 20 ng tBH treated samples compared to controls for DNA denaturing, TdT assay and slot blot hybridization (Fig. 3C). Signal intensity ratios between denatured and non-denatured DNA samples were calculated for tBH treated samples. The signal ratio of denatured DNA to non-denatured DNA was $4.4 \pm 1.1$ (mean ± S.E.) in the cells devoid of tBH treatment, which significantly increased up to $14.9 \pm 3.6$ with increasing concentrations of tBH (Fig. 3D).

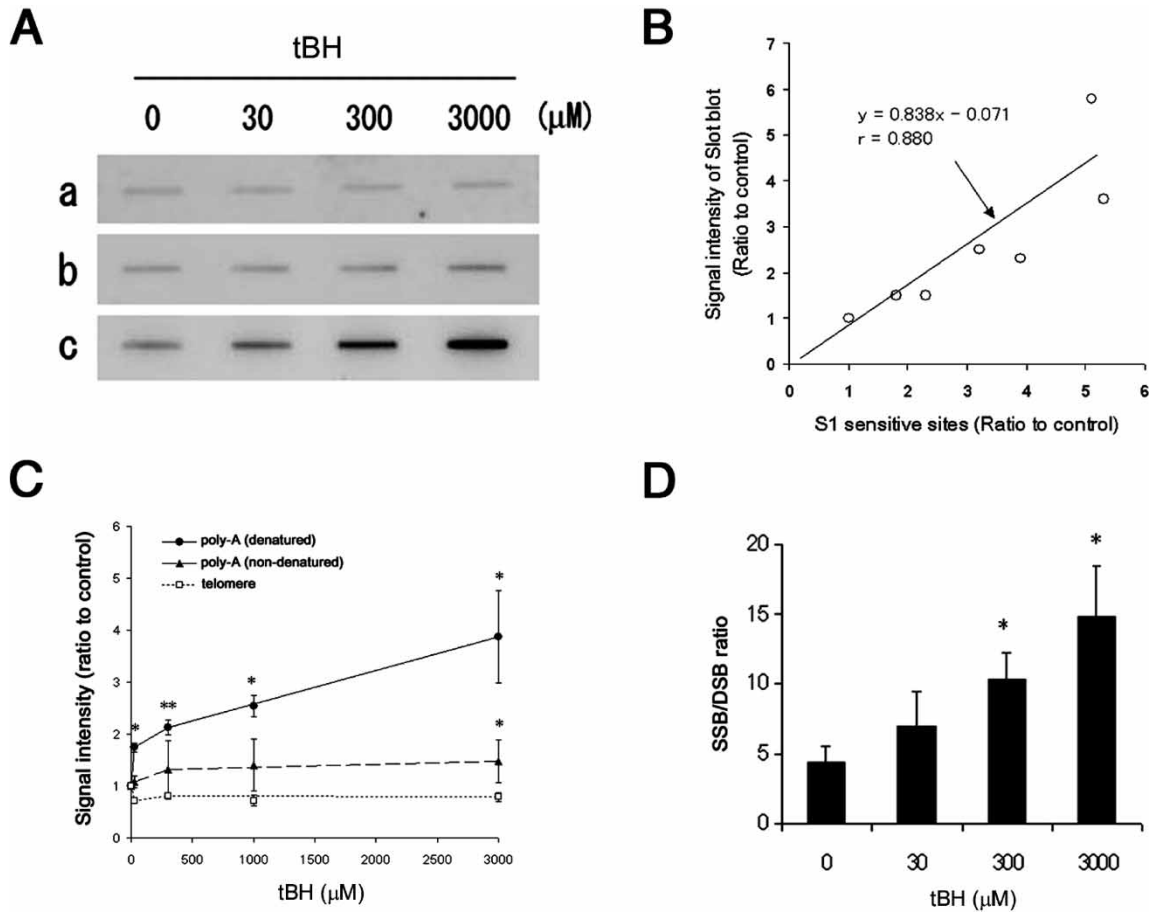


FIGURE 3 (A) Slot blot analysis of DNA from ARPE-19 cells treated with tBH. (a) 200 ng of non-denatured DNA treated by TdT and hybridized with telomere probe. (b) Same blot hybridized with oligo-dT probe. (c) 20 ng DNA samples denatured prior to TdT treatment and hybridized with oligo-dT probe. (B) Data plots for comparing the signal intensities of 20 ng denatured and TdT treated DNA from the cells treated with tBH (ratio to control) to the amount of S1 sensitive site (ratio to control) calculated by gel electrophoresis analysis. (C) Signal intensity of 20 ng DNA samples from tBH treated cells, labeled with poly-A tail by TdT. (●), DNA was denatured prior to TdT treatment and hybridized with oligo-dT probe. (▲), DNA was not denatured before TdT treatment and hybridized with oligo-dT probe. (□), DNA was denatured before TdT treatment and hybridized with telomere probe. (D) Ratio of the signals between denatured DNA (SSB) and non-denatured DNA (DSB) followed by TdT treatment in tBH treated cells. For (C) and (D), values are shown as mean ± S.E. of three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$.

## DISCUSSION

We have demonstrated a new approach to detect DNA strand breaks, which combines DNA denaturation, a TdT labeling assay and slot blot hybridization. The signals of non-denatured DNA samples treated with TdT and probed by oligo-dT were faint unless they were forcibly fragmented by sonication, likely representing the genomic amount of DSBs. In contrast, DNA denaturation separates double strands and exposes all nicks as potential TdT substrates, allowing TdT to detect the majority of SSBs as well as DSBs. This method detected a remarkable increase in SSBs in 20 ng DNA from human RPE cells treated with tBH, while only a mild increase in DSBs was observed in the same samples under non-denaturing conditions.

TdT mediated dUTP nick end labeling (TUNEL) is widely used to detect apoptotic cells, which detects endonuclease-digested genomic DNA.[11] The ability of TdT to detect SSBs, though limited, was suggested by some investigators.[12,19] The increase in labeling intensity in non-denatured DNA from tBH treated cells may not only reflect an increase in DSBs but also partially measure some SSBs induced by oxidative stress. Although heat denaturation may induce artificial strand breaks, artifacts unlikely affect the results of TdT labeling, since the signal intensity of each data point in slot blots is well correlated to the result of gel electrophoresis after an S1 nuclease assay in tBH treated cells. The signal ratio between denatured and non-denatured DNA may approximate the SSB/DSB ratio in genomic DNA, which significantly increases with tBH treatment. Interestingly, the SSB/DSB ratios in cells under physiological culture conditions evaluated by slot blot analysis are consistent to previous reports, even though investigators used different cell lines and quantifying techniques.[20,21]

DNA polymerase-1 nick translation labeling is known to detect most SSBs as well as DSBs,[10] which is useful for detecting early apoptotic or the oxidative stress damage in DNA.[12,13] However, it is difficult to distinguish signals attributed to SSBs from those of DSBs using this method. A comparison of gel electrophoretic patterns of genomic DNA with and without S1 nuclease digestion is an alternative for calculating SSB/DSB ratios. However, electrophoretic comparisons require higher sample volume (up to 1 μg of DNA)[17,22] than slot blot hybridization (up to 20 ng of DNA ≒ 1000–2000 cells). Neutral and alkaline elutions are widely used to quantitate DSBs and SSBs, respectively, however, the variability and inconsistency of elution results remains to be solved.[23]

Further study will confirm whether DNA denaturation, TdT labeling and slot blot hybridization could detect DNA strand breaks in other cell lines. An added advantage to this method is its ability to quantitate oxidative stress-induced DNA strand breaks, this method is composed of simple techniques and demonstrates enough sensitivity for quantitative assessment of cellular SSBs as well as DSBs, especially in small samples.



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