An *O*-Hydroxylamine-Coupled Alkaline Gel Electrophoresis Assay Demonstrates an Accumulation of Real Single Strand Breaks and Intact AP sites in Base Excision Repair Deficient Cells

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Abstract

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(Under the direction of Dr. James A. Swenberg and Dr. Jun Nakamura)

Single strand breaks (SSBs) are among the most frequent DNA lesions caused by endogenous and exogenous agents. The most utilized alkaline-based assays for SSB detection frequently give false positive results due to artifactual SSBs arising from cleaved alkali-labile sites. Here we developed a specific SSB assay using alkaline gel electrophoresis (AGE) coupled with an *O*-hydroxylamine, *O*-(Tetrahydro-*2H*-pyran-2-yl)hydroxylamine (OTX). OTX stabilizes AP sites to prevent their incision during alkaline DNA denaturation. DNA from DT40 and isogenic polymerase β null cells exposed to methyl methanesulfonate were applied to the OTX-coupled AGE assay. The detection of true SSB formation was observed in each cell line with significantly greater formation observed in the null cells. Furthermore, a modification of the assay demonstrated the accumulation of intact AP sites in genomic DNA from both cell lines. OTX use represents a facile approach for assessing SSBs, whose benefits may also be applied to other established SSB assays.

To my snack time buddies: Esra, Brian, Mitch, Sujey and Kristina. Without you, I would have never survived this ordeal. Also, to my family who love me flaws and all.

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List of Abbreviations

AGE – Alkaline gel electrophoresis

ALS – Alkali-labile site

AP site - Apurinic/apyrimidinic site

ARP - Aldehyde reactive probe

ASB – ARP-Slot Blot assay

BER – Base Excision Repair

dRp - Deoxyribosephosphate

MMS - Methyl methanesulfonate

N7-meG – *N7*-methylguanine

OTX – *O*-(Tetrahydro-*2H*-pyran-2-yl)hydroxylamine

OTX-AGE – OTX-coupled alkaline gel electrophoresis

Pol β – Polymerase beta

SSB – Single strand break

Chapter 1

Introduction

Thousands of single strand breaks (SSBs) are believed to arise in cellular DNA daily from endogenous and exogenous sources as well as during DNA repair processes, such as base excision repair (BER) (1). If unrepaired, these lesions threaten genetic integrity through their potential conversion to lethal double strand breaks during DNA replication. Therefore, repair of SSBs is critical to the cell. BER is a primary response pathway for the repair of deleterious DNA lesions including non-bulky DNA adducts, apurinic/apyrimidinic (AP) sites, and SSBs. In its simplest form, BER proceeds as follows: 1) removal of a damaged base through *N*-glycosylic bond cleavage by a mono-functional DNA glycosylase with AP site formation; 2) incision 5' to the AP site by a Type II AP endonuclease, resulting in a SSB with 3'-hydroxyl and 5'-deoxyribosephosphate (5'-dRp) margins; 3) removal of the 5'-dRp and synthesis of the missing nucleotide by DNA polymerase beta (Pol β); 4) and strand ligation by a ligase (2). Efficient BER is imperative because of the inadvertent generation of SSBs as pathway intermediates. While SSBs are one of the most frequent DNA lesions, well-characterized assays with a high specificity for SSBs are lacking.

The ability to detect SSBs has generated much interest for assessing genotoxicity. Alkaline sucrose gradient sedimentation, alkaline filter elution and gel electrophoresis, specifically the single cell gel electrophoresis (comet) assay, are principal methods that have been utilized over the years to detect SSBs (3). Alkaline sucrose gradient sedimentation relies on centrifugation to separate DNA fragments of various sizes (4). Often cells used in

these assays are radioactively labeled to allow for measurement of DNA within fractions of the sucrose gradient. The radioactivity of each fraction is compared to the total radioactivity of the gradient and plotted against fraction number to create a profile used to illustrate extent of damage and calculate break frequency through mathematical modeling. The initial method was limited in sensitivity with the capacity to detect damage with radiation doses > 50 Gy [1 Gy introduces 0.31 breaks per 10^9 Da of cellular DNA or ~1000 SSBs per cell (5)]; however, further refinement of the technique has allowed detection of damage within the 1 Gy range (6). The desire to increase sensitivity in detecting SSBs led to the development of the alkaline filter elution assay. The basis behind alkaline filter elution is that the rate in which single stranded DNA will pass through a membrane filter is dependent on the length of the strand; therefore, the more SSBs present the faster the elution time (7). This method may be performed either with radioactivity or through fluorometric means but requires a large amount of cells. The measurement of DNA within collected fractions of the elution is used to determine the elution rate. Alkaline filter elution can detect damage caused by 0.5-1 Gy (4), providing an increase in sensitivity; however, the reproducibility is poor (4,6). The labor intensive nature of both these methods and the requirement for specialized equipment for the alkaline filter elution influenced the development of easier electrophoretic methods. The most commonly used of the electrophoretic methods is the comet assay. This method allows for the detection of damage at the single cell level through embedding cells within an agarose matrix, lysing them, and performing electrophoresis directly on the nuclei that remain (8). The range of damage detectable by the comet assay has been reported to be 0.2-10 Gy equivalents (5). The ease and relative inexpensiveness of the comet assay has led it to become a popularly used SSB assay (9-10).

The use of alkaline conditions in these assays is intended to denature DNA allowing for better detection of SSBs. However, the presence of alkali-labile sites (ALSs) within the DNA, such as AP sties, can be cleaved by the alkaline conditions used leading to artifactual SSB formation. The resulting overestimation of SSB formation may compromise the reliability of data obtained by the alkaline-based SSB assays (9). The potential for such variability has been observed in intra- and inter- laboratory studies involving the comet assay (11-12).

O-Hydroxylamines have been documented to react with the aldehydic group of AP sites to create very stable complexes that are refractory to β-elimination by enzymatic activity (i.e. AP or dRp lyases) or by high pH (13-15). Methoxyamine has been used *ex vivo* as a BER inhibitor (16-18) and *in vitro* as a stabilizer of AP sites for detection of damaged bases (e.g. N7-methylpurines) (19-20) or AP sites (21-22). However, the acidic methoxyamine must be precisely neutralized before and during use to prevent the artifactual formation of AP sites and SSBs under acidic and basic conditions, respectively. Furthermore, the neutralization of methoxyamine may lead to high salt contamination. The presence of salt retards DNA migration during electrophoresis, ultimately leading to an underestimate of SSB formation (23-24). As an alternative to methoxyamine, the *O*-hydroxylamine *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine (OTX) is available in a form that does not require neutralization; and thus, circumvents any resulting salt contamination. Accordingly, we report here the use of a novel OTX-coupled AGE method for the specific detection of SSBs.

Chapter 2

Materials and Methods

Cell Lines and Cell Culture

The DT40 cells and isogenic DT40-derived Pol β null cells were grown in suspension in RPMI-1640 medium without phenol red (Invitrogen) and supplemented with 10% fetal bovine serum (heat inactivated; Sigma), 1% chicken serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin (25). Cell lines were maintained at 39.5 °C and 5% CO_2.

Chemical Exposure and Cell Harvest

DT40 and Pol β null cells were seeded into 100 cm dishes and allowed to divide overnight to reach the density of $1x10^6$ cells/mL. Cells were exposed to a continuous treatment of 1 mM methyl methanesulfonate (MMS; Aldrich) for up to 4 hours. Control cells received 1X PBS for 2 hour. After incubation, cells were harvested, washed with chilled 1X PBS, pelleted, and stored at -80 °C until DNA isolation. Using this protocol, DT40 cells were also incubated in the absence of chemical to obtain a stock of untreated DNA.

DNA Isolation from Cultured Cells

DNA was extracted using a PureGene DNA extraction kit (Gentra Systems, Inc.) with modifications as described previously (26).

AP Site Preparation by Heat/Acid Conditions

AP sites were produced in stock DT40 DNA through exposure to heat/acid conditions. DT40 DNA was incubated in sodium citrate buffer (10 mM sodium citrate, 10 mM NaH_2PO_4, 10 mM NaCl, pH 5) for 5 minutes at 70 °C, followed by rapid chilling on ice to stop the

reaction (13). Depurinated DNA was collected using microcon YM-3 centrifugal filter devices (Amicon Bioseperations). This DNA was then washed several times with HEPES buffer (50 mM , pH 7) supplemented with 1 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO). DNA was finally resuspended in HEPES buffer containing 1 mM TEMPO.

Creation of a pH Gradient through Treatment with NaOH

Heat/acid-treated DT40 DNA was exposed to various concentrations of NaOH (0.1-250 mM) in HEPES buffer (50 mM) at 37 °C for 15 minutes. The samples were isolated, washed, and resuspended as described above.

Measurement of SSB Repair through NAD(P)H Depletion

SSB repair capacity was determined by a real-time colorimetric assay that measures intracellular NAD(P)H levels (27). PARP-1 activation during SSB repair leads to NAD^+ consumption, which can be indirectly monitored through NAD(P)H depletion (28). Briefly, 5×10^3 cells/well were seeded into a 96-well plate. After a 30 minute incubation period, the cells were treated with 1 mM MMS quickly followed by the addition of a dye containing XTT (Sigma) and 1-methoxy PMS (Sigma). For up to 4 hours, the NAD(P)H-dependent conversion of the XTT tetrazolium salt to a yellow colored formazan dye was measured spectrophotometrically. Decreases in intracellular NAD(P)H levels were determined through comparison of values to those of the PBS control.

Measurement of AP Sites through an ARP-Slot Blot Assay

AP sites were measured using an aldehyde reactive probe (ARP; Dojindo Molecular Tehcnology, Gaithersburg, MD, USA) -coupled slot blot technique, previously described (13).

Alkaline Gel Electrophoresis

An AGE protocol (29) with modifications was used to access SSBs and ALSs in DNA from exposed cells. The 0.7% agarose gel was prepared in gel buffer (50 mM NaCl, 1 mM EDTA, pH 7) and deionized water. After forming, the gel was soaked in mild alkaline running buffer (30 mM NaOH, 1 mM EDTA, pH 12.4) for at least 30 minutes. Equal amounts of DNA (5 μg) were incubated for 1 hour at 37 °C in the presence or absence of 30 mM OTX (Aldrich) in 50 mM HEPES buffer. OTX does not require buffering before or during incubation with DNA. Following OTX exposure, the samples were denatured through treatment with an alkaline buffer (100 mM NaOH, 30 mM OTX, 50 mM HEPES, pH 12.8) for 20 minutes at 37 °C. Samples then received loading buffer (10 mM NaOH, 95% formamide (Fluka), 0.05% bromophenol blue, 0.05% xylene cyanol) and were loaded onto the agarose gel. The gel was electrophoresed at a constant voltage (30 V) for 16 hours at 4 °C. At the completion of electrophoresis, the gel was placed in neutralization buffer (400 mM Tris, pH 7.5) for 20 minutes at room temperature. Gels were stained with acridine orange (5 μg/mL) for 1 hour followed by continuous destaining in deionized water. DNA migration was visualized using an Image Station 440CF system (Kodak). The extent of DNA migration was determined using the Alphalmager (University of Delaware) gel documentation software. A profile of integrated intensity values was generated for each lane that consisted of a value for the region > 23.1 kb and the region ≤ 23.1 kb. These profiles were corrected for background by subtracting the integrated intensity values of a blank lane. DNA remaining in the region > 23.1 kb was considered intact while DNA in the region ≤ 23.1 kb was considered fragmented (3). The percent intact and the percent fragment compared to total lane integrated intensity value were determined for each lane. Statistics

were performed using InStat software (GraphPad). Significance was set at $p < 0.05$. Significance within a group (i.e. cell type or OTX treatment) was determined through a One-Way ANOVA followed by the Dunnett multiple comparison post test. Comparison between the groups at one specific time point was performed using the student t-test.

SSB Formation at *N7*-Methylguanine Adducts through Heat/Piperidine Displacement

To visualize the conversion of MMS induced *N7*-methylguanine (*N7*-meG) adducts into SSBs, the AGE assay described above was modified with a piperidine treatment (30). Equal amounts of DNA (25 μg) were incubated for 1 hour at 37 °C in the presence of MMS (0 or 1 mM) and 30 mM OTX (Aldrich) in 50 mM HEPES buffer (pH 7). Treated DNA was washed and collected using microcon YM-3 centrifugal filter devices (Amicon Bioseperations). The treated DNA was then incubated with alkaline buffer (pH ≥ 12.8) that contained either 100 mM NaOH or 1 M piperidine for 20 minutes at 37 °C. This DNA was then washed and collected by centrifugal filter devices, and the concentrations determined by UV. DNA (5 μg) from each sample received loading dye and was loaded onto an 0.7% agarose gel prepared as previously described. Electrophoresis, neutralization, staining, and image analysis were performed as described above.

Chapter 3

Results and Discussion

Alkaline DNA Denaturation and AP Site Cleavage

The use of alkaline conditions in established SSB assays has demonstrated an efficient means to denature and unwind DNA for the detection of the lesions. However, during DNA denaturation at high pH, intact AP sites, one of the major ALSs existing in genomic DNA, are nicked 3' to the lesion by β-elimination (31). To establish the alkaline denaturation condition for this assay as well as address its ability to cleave AP sites, we used isolated, genomic DNA, treated with heat/acid to generate AP sites, exposed to various NaOH concentrations ranging in pH from 10 to greater than 13. This alkaline treatment of the AP site containing DNA was performed prior to mild alkaline (pH 12.4) agarose gel electrophoresis. Samples that received NaOH concentrations with pH levels below 11.9 displayed large concentrations of DNA just below the wells similar to the control sample, which received no NaOH exposure prior to electrophoresis (Figure 1, Lanes 2-4 verses Lane 1). DNA denaturation and unwinding relies on the disruption of hydrogen bonds at pH > 12 (7, 32). This data supports that these concentrations of NaOH (pH < 12) were unable to denature the DNA for efficient electrophoresis. At a pH above 12.2, the amount of DNA just below the wells was observed to decrease with increasing NaOH concentration and pH level (Figure 1, Lanes 5-12). Also, there was an increase in DNA migration compared to the control with these conditions. This demonstrates that DNA denaturation was increasingly

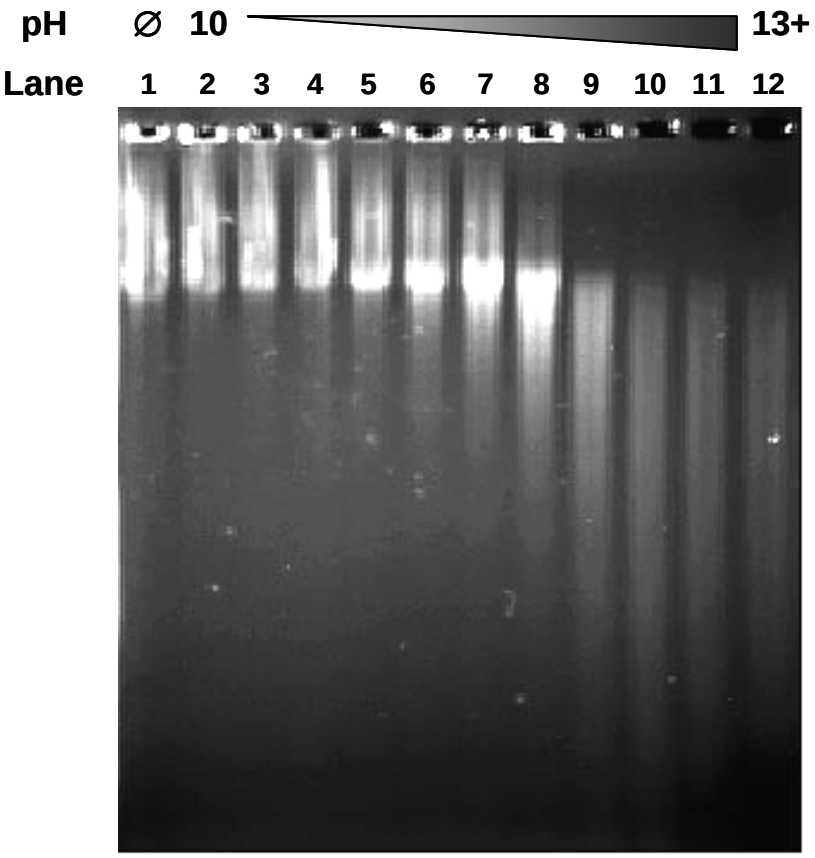


Figure 1. Examining pH effect on DNA alkaline denaturation. Genomic DT40 DNA was exposed to heat/acid conditions for 5 minutes to induce intact AP sites. The DNA was then exposed to various concentrations of NaOH to create a pH gradient (pH 10-13+) prior to being loaded into an agarose gel and electrophoresed under alkaline conditions (pH 12.4). The gels were stained with acridine orange and the image was obtained with a Kodak Image Station 440CF system. Lane 1 – No NaOH (∅); Lanes 2-4 – pH ≤ 11.9; Lanes 5-8 – pH 12.2-12.6; Lanes 9-12 – pH ≥ 12.7.

improved at these NaOH concentrations. Specifically, complete DNA denaturation resulting in efficient migration beyond the untreated control was observed at NaOH concentrations that produce pH levels > 12.7 (Figure 1, Lanes 9-12). Based on these results, we selected 100 mM NaOH (pH 12.8; Figure 1, Lane 10) from that pH range for DNA unwinding in subsequent experiments.

It has been previously reported that AP sites are stable against alkaline cleavage at pH < 12.6 (7, 32). However, under the conditions used in this experiment, the findings indicate that AP sites generated by heat/acid conditions are stable at pH ≤ 11.9 but are increasingly cleaved at pH ≥ 12.2. This suggests that any current SSB assay that relies on alkaline conditions at pH ≥ 12.2 for DNA denaturation may be inducing artifactual SSBs through the cleavage of AP sites, resulting in an overestimate of SSB formation.

OTX Optimization for Protection of AP Sites

With the aim to improve SSB detection under strong alkaline conditions, we assessed the utility of stabilizing AP sites prior to complete unwinding at high pH for subsequent gel electrophoresis. It has been well characterized that *O*-hydroxylamines, such as acidic methoxyamine and neutral ARP, efficiently react with AP sites to generate very stable complexes, which are resistant to β-elimination from Pol β or high pH (13-15). Of the different *O*-hydroxylamine compounds, we selected OTX due not only to its commercial availability at reasonable cost, but also its simple preparation, which requires no titration to neutral pH, thereby eliminating possible high salt contamination. The optimal concentration of OTX needed to protect AP sites from alkaline scission during DNA denaturation was examined in genomic DNA exposed to heat/acid conditions. DNA containing intact AP sites

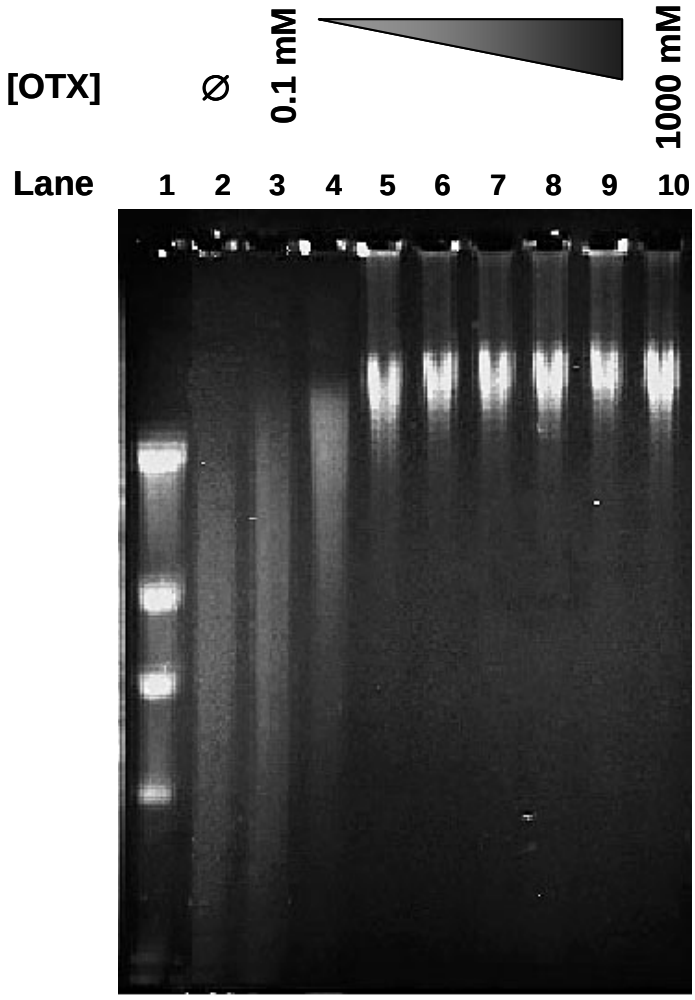


Figure 2. OTX optimization for the protection of AP sites. Genomic DT40 DNA was exposed to heat/acid conditions for 5 minutes to induce intact AP sites. The DNA was then exposed to a concentration gradient of OTX (0.1-1000 mM) prior to denaturation with 100 mM NaOH (pH 12.8) and electrophoresed under alkaline conditions (pH 12.4) in an agarose gel. The gel was stained with acridine orange and the image was obtained with a Kodak Image Station 440CF system. Lane 1 – Marker; Lane 2 – No OTX (∅); Lanes 3-10 – Heat/acid DNA + OTX (0.1, 0.3, 1, 3, 10, 30, 100 and 1000 mM).

was exposed to a concentration gradient of OTX (0.1 – 1000 mM) at 37 ^{o}C for 1 hour prior to denaturation with 100 mM NaOH (pH 12.8) and electrophoresis under mild alkaline conditions (pH 12.4). SSB formation from cleavage of the AP sites during alkaline denaturation was observed as increased DNA migration during electrophoresis. Protection from alkaline cleavage by the reaction of OTX with AP sites was observed as hindered DNA migration during electrophoresis. At concentrations of OTX < 1 mM (Figure 2, Lanes 3-4), the DNA migration was observed to be large with little hindrance compared to the unprotected control (Figure 2, Lane 2). This suggests that minimal protection of AP sites from alkaline cleavage was observed when DNA was pretreated with these concentrations. DNA that is > 23.1 kb is considered intact (3). It was observed that at concentrations of OTX ≥ 1 mM, there was little to no DNA migration beyond the 23.1 kb size marker indicating increased intact DNA resulting from the protection of AP sites against alkaline scission (Figure 2, Lanes 5-10). From the concentrations used to produce these results, 30 mM OTX (Figure 2, Lane 8) was selected as the optimum concentration to protect AP sites from alkaline cleavage and was utilized for the remainder of the experiments in this study.

Accumulation of SSB Repair Intermediates in BER-Deficient DT40 Cells Exposed to MMS

Established methods for detecting SSBs have not been adequately characterized for pH effects on DNA structure and chemistry and also lack a high specificity for SSBs, primarily because of artifactual SSB formation. Potentially, the majority of published data regarding SSB accumulation caused by genotoxic stress may be difficult to interpret due to unintended SSB formation caused by the incision of intact AP sites during strong alkaline conditions. Therefore, we applied the OTX-coupled AGE (OTX-AGE) to address an

accumulation of SSBs as intermediates during a BER response to an acute MMS exposure in the DT40 and Pol β null cells. We first established an accumulation of SSBs in our cell system by using a real-time assay based on NAD(P)H depletion and then compared such observations with those obtained by OTX-AGE. The accumulation of SSBs leads to PARP-1 activation and the consumption of NAD+, which is indirectly monitored in living cells through the measurement of intracellular NAD(P)H depletion using tetrazolium salts, such as XTT, during chemical exposure (27). When exposed to 1 mM MMS for up to 4 hours, DT40 cells displayed a time-dependent decrease in intracellular NAD(P)H levels, suggesting an accumulation of SSBs (Figure 3A). Similarly, the Pol β null cells displayed a time-dependent decrease, but with more extensive depletion occurring as early as 30 minutes of MMS exposure (Figure 3A). These NAD(P)H depletions were mostly protected by the PARP1 inhibitor 3-aminobenzamide (data not shown). Therefore, NAD(P)H depletion in DT40 and Pol β null cells exposed to MMS was due to an accumulation of SSBs.

SSBs formed during the early stages of BER must be processed efficiently to limit the accumulation of these intermediates, which can cause them to potentially become more toxic and mutagenic (33-34) than the preceding base damage: the non-toxic, non-mutagenic *N7*-methylguanine (35) and the toxic *N3*-methyladenine (36). During acute MMS exposure, the NAD(P)H data suggested that BER becomes uncoupled leading to accumulation of SSBs in both the wild-type and Pol β deficient cell lines. A greater accumulation of SSBs detected by more extensive NAD(P)H depletion in the Pol β null cells appear to be due to the inability of these cells to eliminate 5'-dRp by β-elimination (37) or later ligation (38). Based on the results from the NAD(P)H depletion assay described above, DNA was isolated from DT40 and Pol β null cells exposed to 1 mM MMS (1-4 hours) for the direct detection of SSBs by

OTX-AGE. Visually within the gel, MMS (1 mM) was observed to induce a slight increase in DNA migration in DT40 cells exposed from 1 to 4 hours (Figure 3B, Lanes 4, 6, and 8). However, the portion of the samples that was truly single stranded (fragments ≤ 23.1 kb) was insignificant compared to the PBS control except at the 4 hour time exposure (Figure 3C). As may be expected, these data suggest that over extensive exposure times the repair proficient cell line becomes overwhelmed and accumulation of SSBs occurs.

Similarly, the Pol β null cell line also visually displayed a time-dependent increase in DNA migration during the 1 mM MMS exposure (Figure 3B, Lanes 5, 7 and 9). The intensity of DNA fragmentation was slightly lower at 4 hours (Figure 3B, Lane 9) compared to the other time points (Figure 3B, Lanes 5 and 7), probably due to extensive DNA fragmentation and migration in this sample from high SSBs levels. The increase in single stranded DNA (fragments ≤ 23.1 kb) in the MMS treated Pol β null cells was also only observed to be significant at the 4 hour time point (Figure 3C). However, the Pol β null cells displayed significant increases in fragmented DNA compared to the DT40 wild-type at 1-4 hour exposure to 1 mM MMS indicating more SSB formation in the repair deficient cell line (Figure 3C). The difference in lesion formation between the cell lines was as expected due to their difference in repair capacity. However, the lack of significant difference in SSB accumulation observed with the OTX-AGE assay in the Pol β null cells at lower time points of MMS exposure was not expected. The indirect measurement of SSB accumulation with the NAD(P)H depletion assay indicated a pronounced increase in SSBs as early as 30 minutes in the Pol β null cells (Figure 3A). The difference between the assays in demonstrating SSB accumulation calls to attention the differences in their specificity and sensitivity, which may affect their ability to measure SSBs. The NAD(P)H depletion assay is

3A

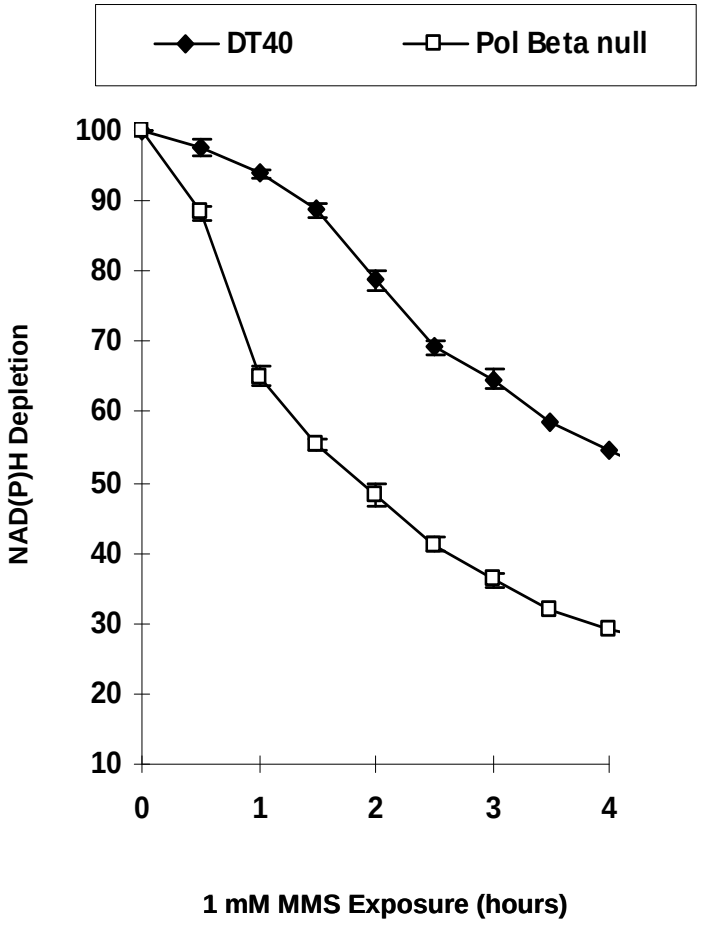
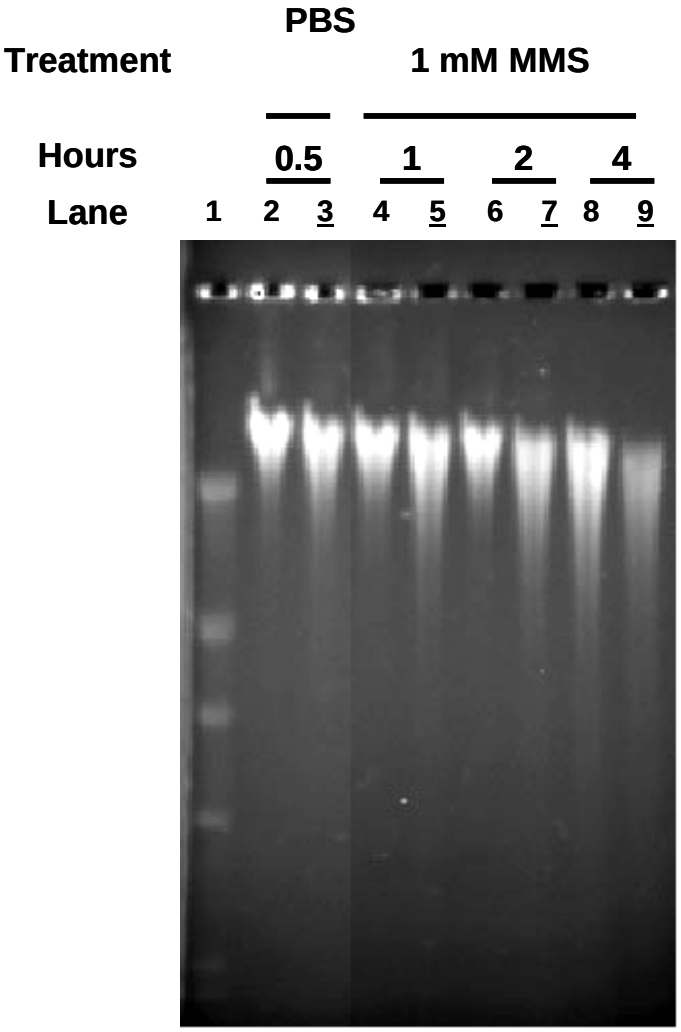
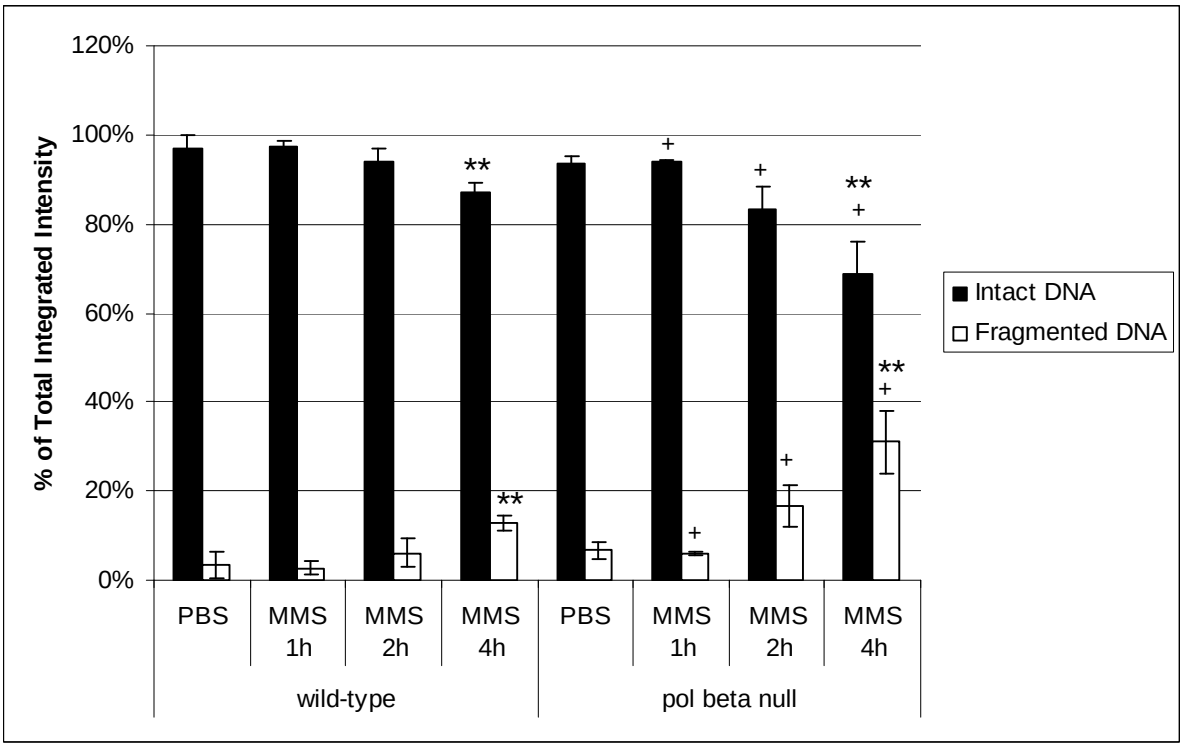


Figure 3. SSB detection in DT40 and Pol β null cells exposed continuously to 1 mM MMS for up to 4 hours. **(A)** Real-time, indirect detection of SSB formation in the cell lines through the monitoring of intracellular NAD(P)H depletion. **(B)** Visualization of SSB formation in the cell lines through OTX-coupled AGE analysis. Even lanes – DT40 cells; Odd lanes with underline – Pol β null cells; Lane 1 – Marker; Lanes 2-3 – PBS control; Lanes 4-9 – 1 mM MMS exposure for 1-4 hours. **(C)** Graphical representation of intact and fragmented DNA in each sample as determined by the Alphalmager gel documentation software. Statistical significance: ** $p < 0.01$, compared to PBS control of the cell line; + $p < 0.05$, compared to the corresponding treatment in the wild-type cell line.

3B



3C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Intact DNA
Fragmented DNA

an indirect measurement of SSBs that relies on monitoring NAD(P)H consumption, which may be sensitive but not specific. The OTX-AGE assay is specific in measuring the actual lesions but its sensitivity in measuring SSBs may be limited in comparison to the NAD(P)H depletion assay.

AP Site Formation in BER-Deficient DT40 Cells during MMS Exposure

BER of alkylated bases leads to the formation of AP sites as pathway intermediates. The affect of Pol β status on the accumulation of AP sites during an acute MMS exposure was examined in DT40 and Pol β null cells using a quantitative slot-blot method (ASB assay) and the OTX-AGE assay. In the ASB assay, both cell lines displayed a time-dependent, significant ($p < 0.01$ for all time points, student t-test) increase in AP sites during continuous exposure to 1 mM MMS compared to control; furthermore, the Pol β null displayed a more massive AP site formation than the parental DT40 cells (Figure 4A). The AP site data at 1 hour were different to what was detected in mouse embryonic fibroblasts deficient in Pol β (39). This discrepancy can be explained by an improvement in DNA extraction through the use of the free radical scavenger TEMPO, which reduces artifactual induction of AP sites, and also differences inherent between mammalian and avian cell lines.

The binding of aldehydic AP sites with an *O*-hydroxylamine, such as OTX, protects them from alkaline scission during AGE. Therefore, preparing DNA samples with and without OTX for AGE allows for the detection of SSBs only (i.e. with OTX treatment) or a combination of SSBs and AP sites (i.e. without OTX treatment). By comparing OTX treated and untreated pairs of DNA samples, a visualization of the extent of intact AP site formation can be obtained with AGE analysis. OTX treatment did not reveal a visual difference in

4A

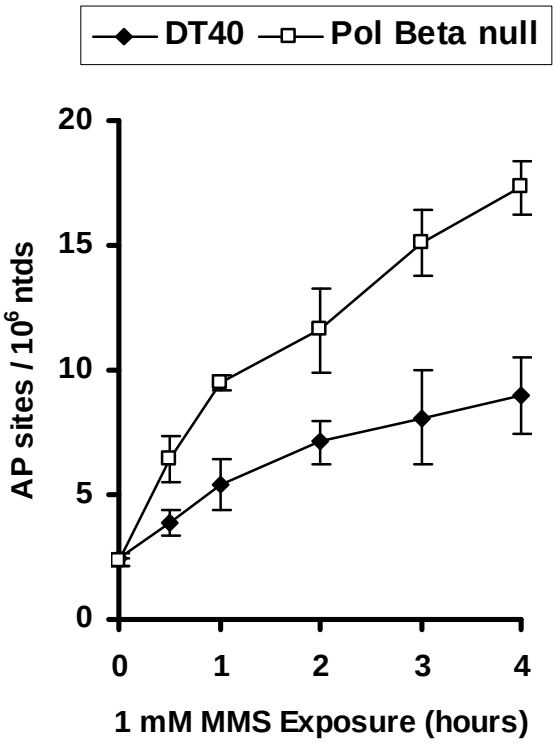
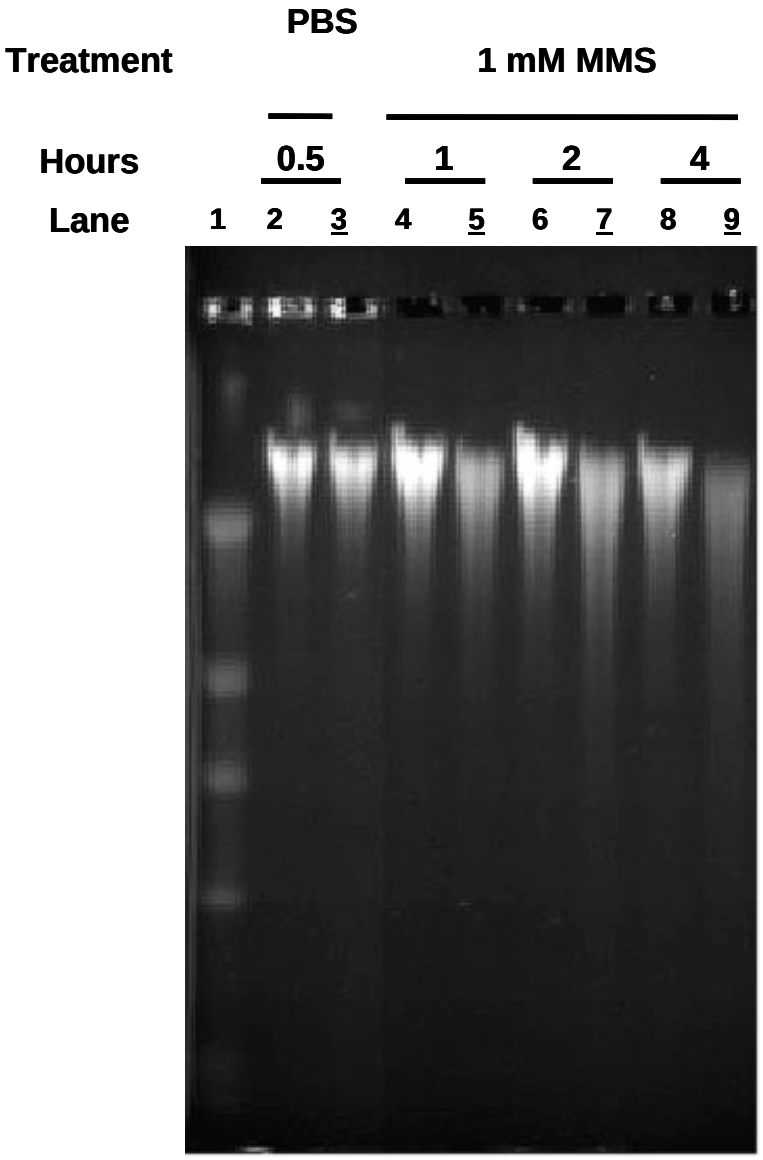
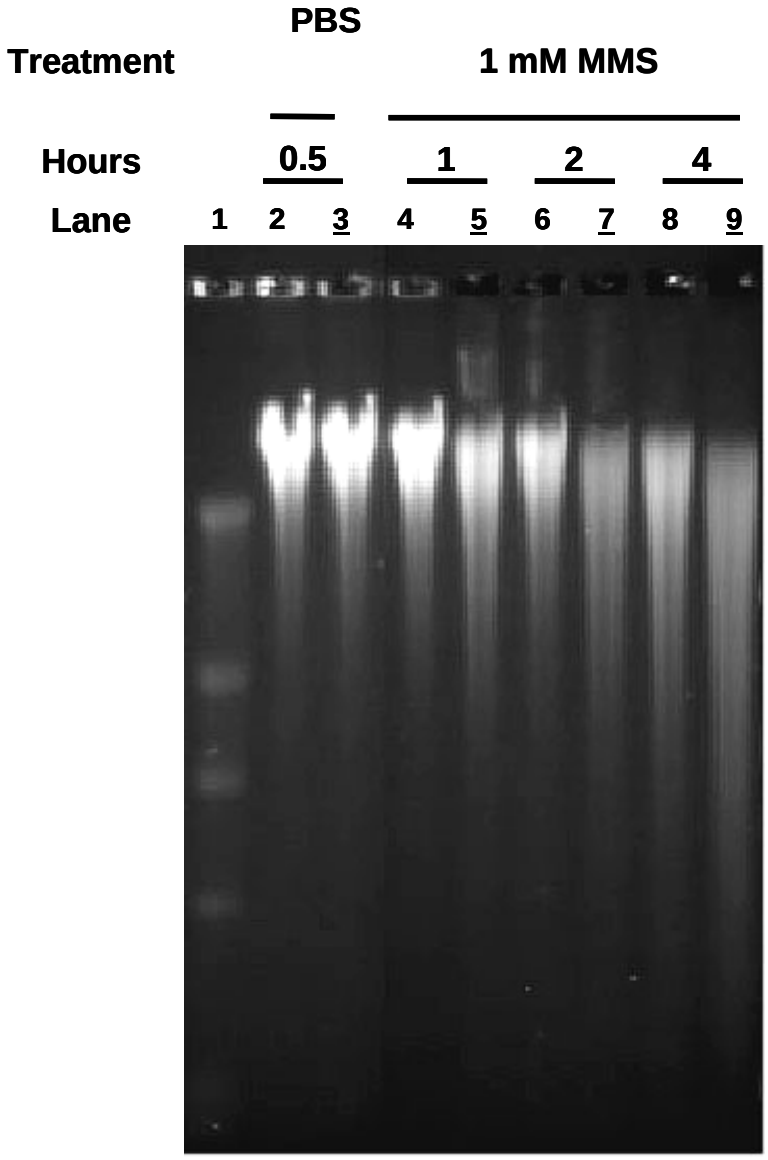


Figure 4. AP site detection in DT40 and Pol β null cells exposed continuously to 1 mM MMS for up to 4 hours. **(A)** Numerical data of AP sites detected through the ASB assay. The mean values represent four independent measurements. Bars indicate SD. **(B-C)** AGE analysis with and without OTX protection. Even lanes – OTX protection; Odd lanes with underline – No OTX protection; Lane 1 – Marker; Lanes 2-3 – PBS control; Lanes 4-9 - 1 mM MMS exposure for 1-4 hours. **(C-D)** Graphical representation of intact and fragmented DNA in each sample as determined by the Alphalmager gel documentation software. Statistical significance: * $p < 0.05$, ** $p < 0.01$, compared to PBS control of the cell line; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ compared to the corresponding OTX treatment.

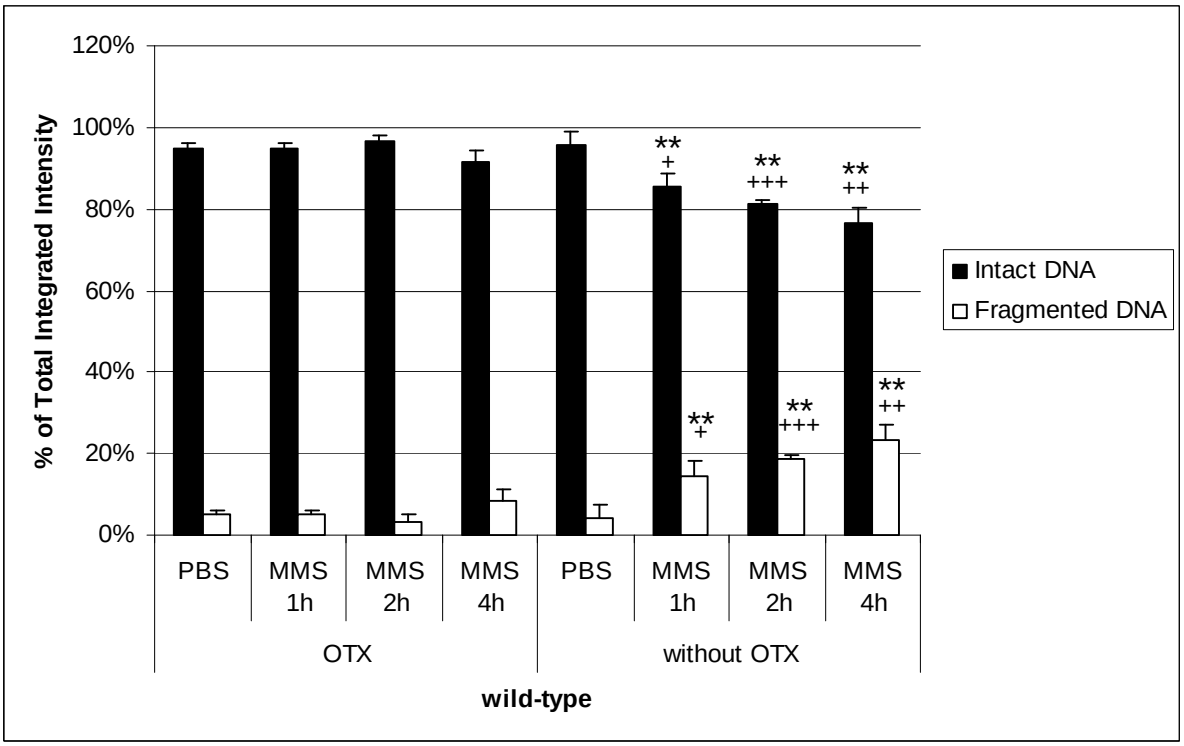
4B: Wild-type



4C: Pol β null



4D: Wild-type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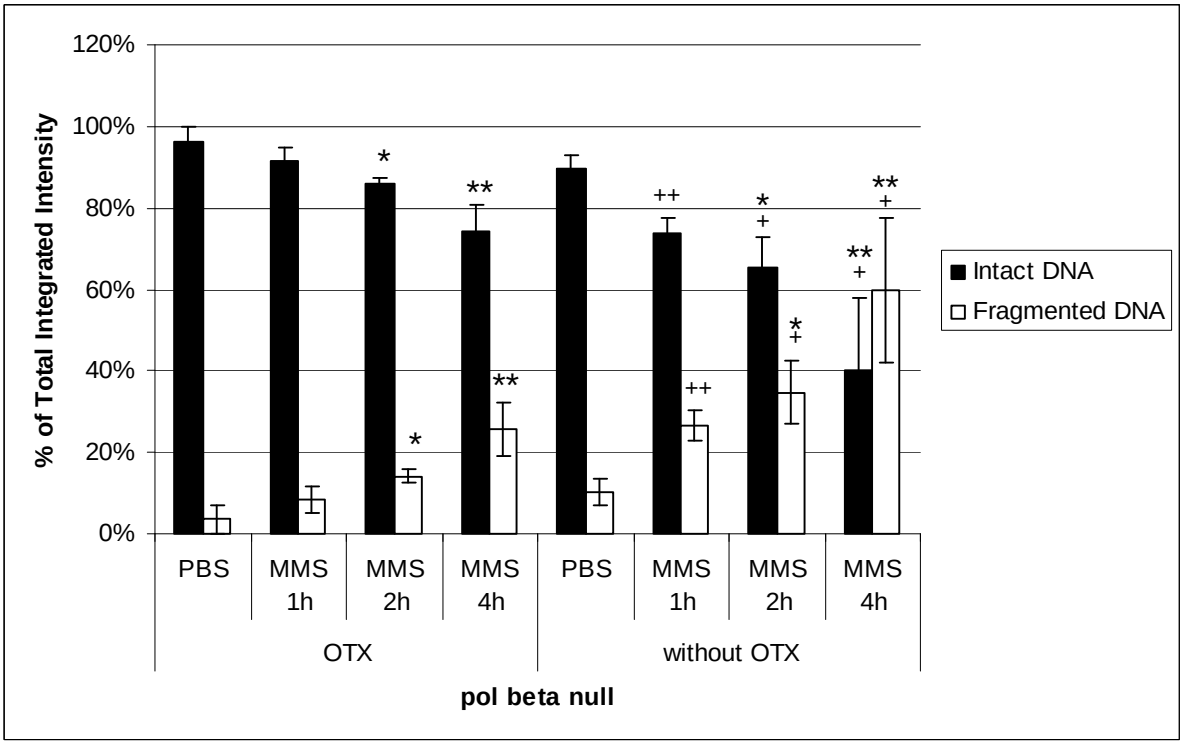
Fragmented DNA

4E: Pol β null


120%
100%
80%
60%
40%
20%
0%
% of Total Integrated Intensity
PBS
MMS 1h
MMS 2h
MMS 4h
PBS
MMS 1h
MMS 2h
MMS 4h
OTX
without OTX
pol beta null
Intact DNA
Fragmented DNA

DNA migration among the control samples of each cell line (Figure 4B-C; Lanes 2-3) and the percent of single stranded DNA (fragments ≤ 23.1 kb) was not significantly different (Figure 4D-E). This suggests that there were low endogenous, intact AP site levels within each DT40 based cell line, which is similar to the low levels of AP sites reported for control cells as determined by the ASB assay. After MMS exposure and pretreatment with OTX, AGE analysis of the DT40 DNA revealed no significant increase in SSB formation over 4 hours of exposure (Figure 4B, even lanes; Figure 4D). However, without OTX pretreatment, a time-dependent increase in DNA migration was visually observed (Figure 4B, odd lanes). This resulted from the cleavage of intact AP sites leading to the significant increase in fragmented DNA when compared to the OTX treated samples at each time point (Figure 4D). In the MMS treated Pol β null cells, both SSB and intact AP site formations were observed to increase in a time-dependent manner (Figure 4C and 4E). These lesions appear to have formed to a greater extent in the Pol β null cells when compared to the DT40 wild-type cells (Figure 4B and 4D), which would be in agreement with the quantitative data obtained by the ASB assay (Figure 4A).

ALSs such as *N7*-methylguanine (*N7*-meG), the predominate lesion formed by monofunctional alkylating agents, are generated during MMS exposure (40). In the absence of OTX, ALSs other than AP sites may contribute to SSBs detected during AGE. To assess this phenomenon, we simultaneously exposed isolated, genomic DNA to MMS (0 or 1 mM) and 30 mM OTX to induce *N7*-meG formation and protect pre-existing or endogenous aldehydic sites from alkaline strand scission during subsequent AGE. As a positive demonstration of the contribution of ALSs to the SSBs detected, we incubated treated DNA with piperidine instead of NaOH during DNA denaturation. The introduction of

heat/piperidine and resulting alkaline pH induced SSBs at *N7*-meG adducts through a series of chemical events involving imidazole ring opening of *N7*-meG, displacement of the resulting formamido-pyrimidine from the deoxyribose backbone with AP site formation, and β-elimination of the AP site with concurrent SSB formation (30). When the MMS exposed DNA received piperidine treatment, a notable increase in DNA migration was observed (Figure 5; Lane 5); however, the increase in DNA migration was not seen in the PBS control that also received piperidine treatment (Figure 5; Lane 4). For MMS treated and control DNA subjected to NaOH induced DNA denaturation, there was no difference in the amount of DNA migration (Figure 5; Lanes 2-3). The increase in migration with piperidine treatment of the MMS exposed DNA demonstrates the presence of the *N7*-meG lesions in these samples. In contrast, the lack of migration observed in the MMS exposed DNA treated with NaOH suggests that this denaturation condition does not cleave the *N7*-meG lesions; thus, these lesions did not artifactual contribution to SSB detection. Together, these data indicated that AP sites were the predominant source of ALSs in the highly methylated DNA analyzed under our conditions.

Overall, these results demonstrated that while the ASB assay provides an entire fraction of AP sites (i.e. intact AP sites and cleaved AP sites), the difference between AGE analysis with and without OTX defines the existence of intact AP sites before incision by either type I or type II AP endonuclease. These data also indicate that MMS causes the accumulation of not only overall AP sites, but also intact AP sites during the exposure of methylating agents, suggesting that incision of AP sites by AP endonuclease 1 can be saturated under massive DNA methylation by MMS.

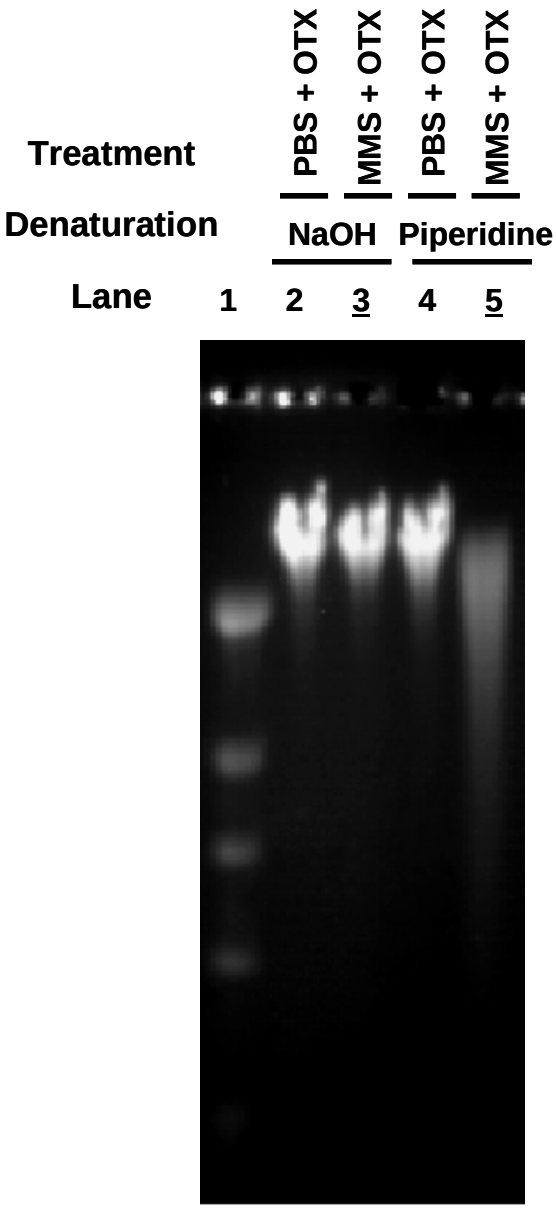


Figure 5. Distinguishing SSBs arising from ALSs other than AP sites. Genomic DNA was simultaneously exposed to MMS (0 or 1 mM) and 30 mM OTX for 1 hour. Treated DNA was then denatured with either 100 mM NaOH or 1 M piperidine for 20 minutes prior to being loaded into an agarose gel and electrophoresis under alkaline conditions (pH 12.4). Even lanes – PBS plus OTX treatment; Odd lanes with underline – MMS plus OTX treatment; Lane 1 – Marker; Lanes 2-3 – NaOH denaturation; Lanes 4-5 – Piperidine denaturation.

Chapter 4

Conclusions

Advantages and Disadvantages of the OTX-Coupled AGE Assay

Through the stabilization of AP sites, the OTX-AGE assay provides a specific means for detecting SSBs. In addition, however, the OTX-AGE assay also offers a number of other advantages. This approach is a relatively easy, time efficient assay based on common electrophoresis techniques. Because it is an electrophoretic method, the equipment used in the assay is often available in laboratories and the chemicals are readily available from commercial sources. Since the OTX-AGE assay uses very little DNA (5 μg per sample), this method can be used in situations where DNA sample amounts are limited. This assay is also versatile because samples analyzed by OTX-AGE may represent either DNA isolated from various tissues or cells after *in vivo* exposures or DNA purified after *in vitro* exposures to genotoxicants; the use of isolated DNA is not be applicable to the comet assay. The rapid generation of results from OTX-AGE is also very attractive. This method could serve as a valuable means for rapidly screening the ability of exogenous and endogenous agents to induce SSBs prior to assessing additional measures of DNA damage.

A main disadvantage of the OTX-AGE assay is that it is semi-quantitative. The images of the gels provides a qualitative measure of SSB formation much like the qualitative nature of the 'comets' produced by the comet assay. Similar to the assignment of tail moment with comet assay software, the use of the Alphalmager gel documentation software to determine percent intact and percent fragmented DNA does provide more measurable

information on the qualitative images of the gels. However, this semi-quantitative measure does not provide information on break frequency. This disadvantage may be overcome thorough the use of a mathematical model reported by Sutherland *et. al.* known as number-average length analysis (41). In generalized terms, this analysis uses the knowledge of DNA length and electrophoretic mobility to determine the average length of DNA within a sample to represent the break frequency.

Further optimization of the OTX-AGE assay, specifically in regards to gel imaging, may prove to be beneficial. The gel images used in this work were obtain from gels that were stained with acridine orange for 1 hour followed by 4 hours of destaining in deionized water. Acridine orange, like the nucleic acid stain ethidium bromide, binds double stranded DNA by intercalating between the stacked bases in the double helix. Also similar to ethidium bromide, acridine orange binds single stranded DNA, but with better sensitivity. Because ethidium bromide binds thorough intercalation between the stacked bases, its sensitivity in detecting single stranded DNA is low. In contrast, acridine orange is able to electrostatically bind to single stranded DNA allowing for better detection. For this reason, acridine orange was selected as the nucleic acid stain. However, the use of other nucleic acid stains may demonstrate further improvement in gel imaging. For example, SYBR Gold has been shown to be the most sensitive stain for DNA with low background (42). Though it is very expensive in comparison to other stains, especially acridine orange, the improvement in gel image may be worth the cost. To further improve gel imaging, the optimal destaining period to reduce background fluorescence and allow for better detection of the DNA may also be determined. Any decrease in background would improve the determination of integrated intensities by the gel documentation software, which relies on the fluorescent

densities to identify the presence of DNA, and would lead to improve analysis. Another optimization for gel imaging and analysis would be the use of an image system that allows for the selection of the specific excitation and emission wavelengths of the nucleic acid stain chosen. The Kodak imager used in this work employs light between 300-400 nm to excite a broad range of fluorochromes. To create more specificity for the fluorochrome used, there are also various emission filters to enhance the image captured by the system. When acridine orange is bound to single stranded DNA, it has an excitation wavelength of 460 nm and an emissions wavelength of 650 nm. Though the Kodak imager may have been able to excite the bound acridine orange, this was not at the optimum wavelength. Similarly, the Kodak imager did not have an emissions filter for wavelengths above 627 nm; thus, limiting the quality of image produced when using acridine orange with this system. It is expected that improved images would be obtained for analysis with an imager that had a broader range of excitation wavelengths and emission filters to allow for capturing images specific to the nucleic acid stain used.

Overall, the OTX-AGE assay may be most improved thorough the determination of the best sample size to obtain significant results. A sample size of 3 was used throughout this work, which produced acceptable standard deviations. However, some of the results within the DT40 cells were not as expected. It may be asked whether this was due to the biology or the limited sample size. For example, the lack of significant difference in SSBs compared to the control in the Pol β null cells exposed to MMS at the 1-2 hour time points (Figure 3C) was unexpected when an accumulation of these lesions had been established at these time points in the NAD(P)H depletion assay (Figure 3A). Perhaps an increased sample size may have demonstrated the same accumulation, bringing those assays into agreement. Finding

the optimum sample size would also reduce variability in performing a gel based assay. It is expected that samples with the same treatments would produce similar results regardless of when they are processed through AGE. A comparison of the 4 hour MMS exposure in the wild-type cells that also received OTX pretreatment in Figure 3C and Figure 4D demonstrated a difference in SSB accumulation for the same treatment. Whereas the accumulation was found to be significant when compared to the control in Figure 3C, this was not true for the same treatment group in Figure 4D. An increased sample size may help to reconcile this variability in response, allowing for a clearer understanding of the biology within the sample.

Potential Overestimate of SSB Formation by Current Assays

The ability of alkaline to cleave AP sites is well established in the literature (31) and is demonstrated in this work in Figure 1. A majority of current SSB assays rely on alkaline treatment for DNA denaturation, regardless of its ability to induce AP site scission leading to artifactual SSB detection. In this work, the ability of OTX to prevent AP site cleavage and thus enhance detection of true SSBs was demonstrated. In addition, the data also presents evidence for the agreement that SSB data reported in the literature to date may represent a gross overestimate of formation. In both DT40 based cell lines, the presence and absence of OTX pretreatment in DNA exposed to 1 mM MMS demonstrated the presence of intact AP sites (Figure 4D-E). The significant increase in fragmented DNA in samples without OTX pretreatment compared to those with pretreatment was observed by the 1 hour time exposure in both cell lines, suggesting an increase in cleaved AP sites by this time point (Figure 4D-E). In comparison, the significant increase in fragmented DNA in samples that received OTX pretreatment compared to their PBS control was not observed in the DT40 wild-type and was

observed in the Pol β null cell but not until the 2 hour time exposure (Figure 4D-E). This suggests that SSBs had not accumulated in the DT40 wild-type cells, but had accumulated in the Pol β null cell by the 2 hour time point. The ability of AP sites to contribute to fragmented DNA earlier than the detection of the true SSBs in these cells stresses the need to prevent them from confounding SSB detection data.

Application of OTX to Other SSB Assays

The use of OTX may benefit other established alkaline-based SSB assays, such as the comet assay. The inclusion of OTX in the comet assay could protect AP sites within the cells during processing and electrophoresis while still allowing the use of high alkaline conditions; thus, improving the assays specificity without a reduction in its sensitivity. As a preliminary test of this hypothesis, Chinese hamster ovary AA8 cells were exposed to 1 mM MMS for 0-10 minutes and then processed them through a standard comet assay protocol. As expected, there was a significant increase in tail moment with MMS treatment (Figure 6). After establishing the presence of damage with MMS treatment, the experiment was repeated with cell exposed only to MMS for 10 minutes using the same standard protocol but with an additional step between the lysis and denaturation steps where embedded cells were exposed to either PBS or 300 mM OTX for 1-4 hours. The aim was to allow the OTX to react with the DNA after the lysis of the cell so that any AP sites present may be stabilized prior to alkaline unwinding and electrophoresis. Without OTX exposure, MMS exposed cells demonstrated a significant increase in tail moment over the 4 hour incubation in PBS indicating the induction of artifactual damage that would interfere with the measurement of real SSBs (Figure 7). With the addition of OTX, a significant decrease in tail moment was observed in these samples when compared to the same treatments without OTX as well as to

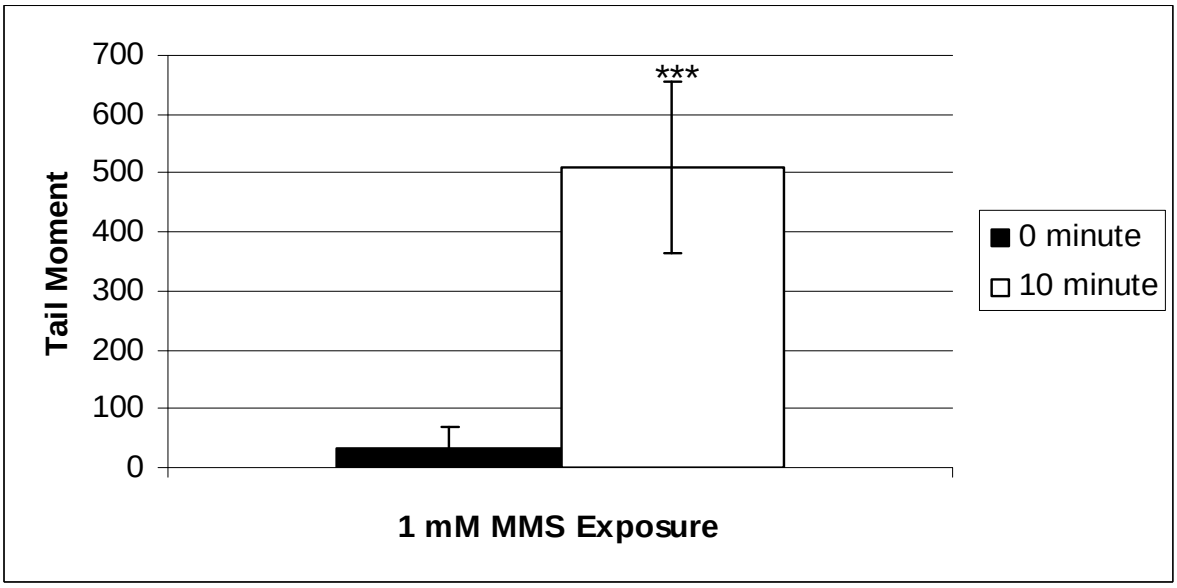


Figure 6. Difference in tail moments resulting from MMS exposure. Chinese hamster ovary AA8 DNA was exposed to 1 mM MMS for 0-10 minutes and then processed through the comet assay. Data is shown as mean ± standard error of means. Statistical significance: *** $p < 0.001$, compared to the control.

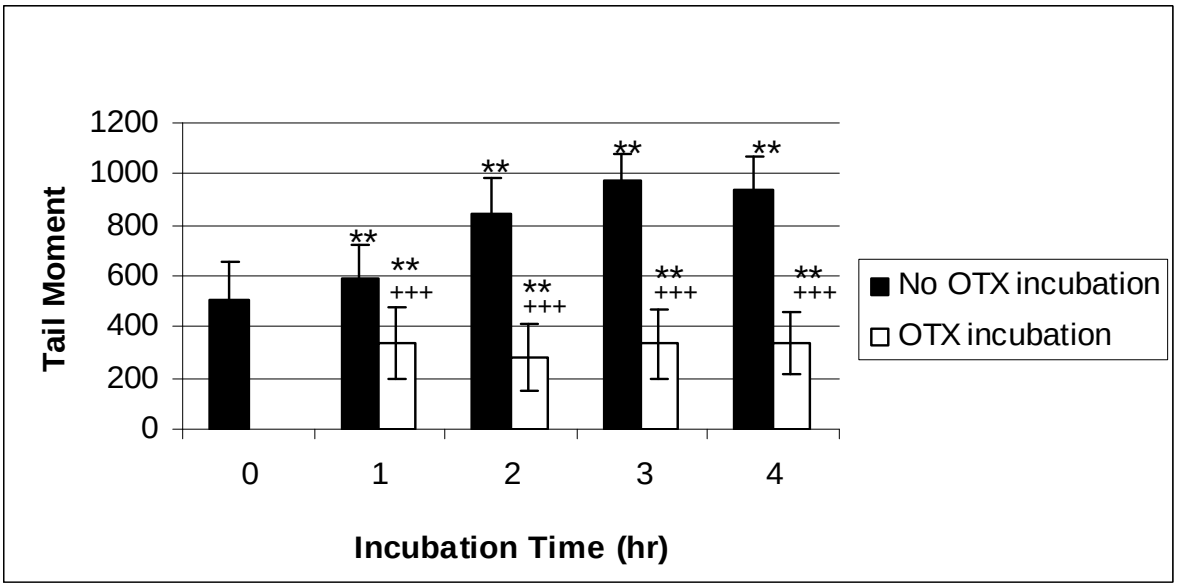


Figure 7. Difference in tail moments resulting from MMS exposure ± OTX protection. Chinese hamster ovary AA8 cells were exposed to 1 mM MMS for 10 minutes then processed through a modified comet assay that contained a PBS or 300 mM OTX treatment after cell lysis. Data shown as mean ± standard error of means. Statistical significance: ** $p < 0.01$, compared to the 0 hour incubation/no OTX protection control; +++ $p < 0.001$, compared to the corresponding incubation times that received no OTX protection.

the 0 hr control; and this tail moment was stable of the 0-4 hour OTX incubation period (Figure 7). This suggests that OTX inclusion was able to reduce the affect of artifactual damage and provide consistent protection over comet processing. These preliminary data support the proposal that OTX addition to the comet assay would be beneficial and further development of this application may be of great interests to researchers who rely on the comet assay. It also stresses, along with the AGE data presented throughout this work, the potential overestimation of SSB formation reported throughout the SSB literature and the need to consider this carefully when using that type of data.

Closing Summary

In conclusion, the present study reports the development and validation of a rapid and specific assay for the detection of true SSBs. Based on agarose gel electrophoresis techniques, OTX provides protection of AP sites from alkaline strand scission while allowing for complete denaturation of DNA. Such aspects are both desirable for the specific and sensitive detection of real SSBs. As a proof of principle, this assay was applied to DNA from MMS treated DT40 and Pol β null cells. As expected, Pol β null cells displayed higher SSB formation. To our knowledge, this is the first time the accumulation of real SSBs, without potential confounding by ALSs, has been demonstrated in BER deficient cells. The use of OTX also allowed for the detection of intact AP sites within both cell lines, which suggests that APE1 may become saturated during extensive MMS exposure. The OTX-AGE assay offers many benefits but also presents disadvantages which may be resolved with further optimization. Due to its ability to reduce artifact through the protection of AP sites, it is proposed that other SSB assay may benefit from the addition of OTX to improve their specific for SSB detection.

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