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# DNA Damage and mRNA Levels of DNA Base Excision Repair Enzymes Following $H_2O_2$ Challenge at Different Temperatures

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## 1. Introduction

Our cells are continuously exposed to endogenous and exogenous oxidizing agents that can damage DNA leading to disruption of transcription, translation, and DNA replication (Davies, 2000). Accordingly, protective mechanisms, including the presence of cellular antioxidants and induction of enzymatic repair of damaged lesions, are necessary in order to survive in this oxidizing environment. When damaged DNA lesions are not prevented or properly repaired, they can cause mutations that increase the risk of degenerative diseases.
The baseline level of oxidative damage associated with normal cellular processes has been estimated as high as 1 base modification per 130,000 bases in nuclear DNA (Davies, 2000). However, high levels of reactive oxygen species (ROS) result in an increase in modified DNA levels. Overall more than 20 modified DNA base lesions have been identified, including 8-oxoguanine which is the most abundant DNA adduct (Cooke et al., 2003). In addition, ROS can attack DNA, generating strand breaks, sugar damage and DNA-protein cross-links. A single strand break (SSB) is a discontinuity in the sugar-phosphate backbone of one strand of a DNA duplex leaving modified ends which inhibit or block DNA polymerases and DNA ligases (Caldecott, 2001). SSBs can be produced directly from ROS attack, indirectly from DNA repair processes, by direct disintegration of deoxyribose, or by abortive DNA topoisomerase 1 activity (Dianov & Parsons, 2007; Leppard & Campoux, 2005).
DNA repair mechanisms to maintain the genomic integrity have been described. DNA base lesions and single strand breaks resulting from ROS-induced oxidative attack are mainly repaired through the base excision repair (BER) pathway (reviewed in (Caldecott, 2003; de Murcia & Menissier de Murcia, 1994; Dianov & Parsons, 2007; Wilson, 2007)). In order to repair DNA base lesions, BER is initiated by specific DNA glycosylases. For example, human 8-oxoguanine DNA-glycosylase 1 (hOGG1), a bifunctional glycosylase, recognizes and cleaves 8-oxoguanine and also catalyzes 3′ of the abasic site (AP site). Following this initiation step, AP-endonuclease I (APE1) cleaves the AP site making a gap between the DNA 3′-OH and the 5′-phosphate. The SSBs, produced either directly from ROS attack or indirectly from DNA repair processes, can then be recognized by the enzyme poly (ADP-ribose) polymerase-1 (PARP1). Binding of PARP-1 to the AP sites stimulates the formation of poly (ADP-ribose) polymers and dissociation of PARP-1 from the DNA-recruiting BER

proteins at the damage site. DNA polymerase β fills the gap by DNA synthesis. Finally the resulting nick is sealed by DNA ligase, completing the short-patch repair pathway. In this pathway X-ray cross-complementing 1 (XRCC1) plays a major role in facilitating the interaction among the proteins involved in the BER pathway such as APE1, DNA polymerase β and DNA ligase III (Caldecott, 2003). Depending on the nature of AP sites, some AP sites are repaired by the long-patch repair pathway requiring different enzymes including flap endonuclease and DNA ligase I. Even though the basic DNA repair mechanisms are well described, recent evidence suggests that DNA repair mechanisms are quite complicated with more than 100 proteins involved in the repair of various lesions (Wood et al., 2001).

Deficiencies in DNA repair systems have been shown in several types of cancer (Langland et al., 2002; Lynch & Smyrk, 1996; Marchetto et al., 2004). However, whether such deficiencies in DNA repair enzymes are associated with single nucleotide polymorphisms (SNPs) is still arguable.

Several studies have examined the DNA repair capacity of different cells upon exposure to environmental agents such as oxidants or antioxidants (Astley et al., 2002; Collins et al., 1995, 2003; Torbergsen & Collins, 2000). Most studies have either only monitored DNA damage or determined the mRNA expression levels of DNA repair enzymes, mostly hOGG1, in order to elucidate the role of oxidants or antioxidants on DNA repair activity. However, monitoring only DNA damage for DNA repair kinetics reflects more global effects instead of specific aspects of repair (Berwick & Vineis, 2000). In addition, the exact relationships between oxidative stress, DNA damage and induction of the mRNA expression levels of repair enzymes including hOGG1 is unclear (Hodges & Chipman, 2002; Kim et al., 2001) although hOGG1 has been shown to be inducible responding to various oxidative conditions (Kim et al., 2001; Lan et al., 2003).

As these previous studies indicate, multiple methods and markers of DNA damage and repair may be needed in order to explain molecular responses to DNA damaging agents. Furthermore, information is still lacking about the rate of DNA repair immediately following treatment with oxidants or antioxidants; this information may be important in determining steady-state damage levels following induction of oxidative stress (Collins & Harrington, 2002). Therefore the current study was undertaken to better understand the relationship between cellular DNA damage and induction of mRNA expression of repair enzymes following acute oxidant treatment using Caco-2 cells (human colon cancer cells). Oxidant ($H_2O_2$) treated cells were monitored over an extended recovery period, and both DNA damage levels and mRNA levels of several DNA BER enzymes (hOGG1, APE1, PARP1, XRCC1) were quantified over time in order to better understand DNA repair kinetics and the molecular responses to an oxidative DNA damaging agent.

## 2. Materials and methods

### 2.1 Chemicals and reagents

Caco-2 cells were generously donated by Dr. Bo Lonnerdal (University of California, Davis). Hydrogen peroxide, penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO). Minimum essential medium (MEM) with Earl's salts, including 1-glutamine and 0.25% trypsin-EDTA solution, were from Gibco (Invitrogen, Carlsbad, CA). Fetal bovine

serum was obtained from Gemini (West Sacramento, CA). For the single cell gel electrophoresis assay, a commercial kit was purchased from Trevigen Co. (Gaithersburg, MD). For real time PCR, reverse transcription kits and SYBR Green PCR master mix were purchased from Applied Biosystems (Foster City, CA) and Roche (Mannheim, Germany), respectively.

### 2.2 Cell culture and treatments

Caco-2 cells were grown in MEM, supplemented with 10% (v/v) fetal bovine serum, L-glutamine, 1% penicillin and streptomycin (10 units/mL and 1 mg/mL respectively) at 37°C in a humidified environment composed of 5% $CO_2$ and 95% air; the growing medium was changed every two days. Cells were subcultured at 80-90% confluency. After seeding onto a 100 mm cell culture plate with a density of $5\times10^6$ cells/plate, cells were grown for one day and treated with fresh hydrogen peroxide (100 μM) for 30 min at 37°C, a physiologically relevant temperature, or for 10 min at 4°C, a condition where DNA repair should be minimized. After washing with phosphate buffered saline (PBS), cells were incubated in growing medium (including serum) at 37°C for up to 5 h. For the cells treated with oxidant at 37°C, they were further incubated for 8 hours in order to confirm the mRNA expression pattern of some DNA repair genes. Some cells were collected for comet assay and the others were collected for RNA extraction. Each experimental treatment was performed in duplicate on 3 different days.

### 2.3 Measurement of DNA damage

After incubation in growing medium for 0-5 h, cells were harvested with trypsin-EDTA solution, washed twice with ice-cold PBS, and cell viability was determined with the trypan blue exclusion test. The single cell gel electrophoresis (comet assay) procedure was based on methods of Singh et al. with slight modifications (Singh et al., 1988). A commercial comet assay kit was used to measure strand breaks following the manufacturer's protocols. Briefly, cells were diluted with PBS in order to have a cell density of $1\times 10^5$ cells/mL and embedded into low melting point agarose on comet slides. Embedded cells were lysed in lysis solution (including 1% sodium lauryl sarcosinate) for 1 hour and unwound in alkaline solution (300 mM NaOH, 1 mM EDTA, pH > 13). Subsequently, electrophoresis was performed for 30 min at 300 mA. Cells were neutralized by washing with water, dried following immersion in ethanol, and kept at room temperature in the dark until silver staining. Silver stained cells were imaged using a Nikon E600 with a Leica LEI-750 camera.

Images were analyzed by measuring % tail DNA of each cell using CometScore software (version 1.5, www.autocomet.com). Cells (75 total) were collected from 2 slides per treatment and the whole procedure for DNA damage measurement was repeated three times independently. Slides were coded and counted blindly; after imaging and counting, slides were decoded in order to quantify differences among samples.

### 2.4 Measurement of expression of DNA repair enzymes

#### 2.4.1 Total RNA extraction

Total RNA was extracted from Caco-2 cells using Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The concentrations of extracted RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington,

DE) with the quality of RNA determined from the absorbance ratio of $A_{260}/A_{280} > 1.8$ and confirmed by gel electrophoresis. Extracted RNA was preserved at -80°C until used. cDNA was synthesized using 5 μg total RNA, oligo $d(T)_{16}$ primers, and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). Reverse transcription was performed by following the manufacturer's protocol.

### 2.4.2 Real-time quantitative RT-PCR (qRT-PCR)

In order to detect DNA repair enzyme genes (hOGG1, APE1, PARP1 and XRCC1) and β-actin (used as a reference gene), qRT-PCR was performed using SYBR Green PCR Master Mix reagents (Roche, Mannheim, Germany) on a PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Specific primers for each gene are shown in Table1. Real time quantitative RT-PCR (real time qRT-PCR) was performed through the amplification for 40 cycles of 95°C (30 sec), 58°C (30 sec) and 60°C (1 min) after activation of enzyme at 95°C (10 min). The data were normalized using β-actin as an internal standard. Relative fold changes were calculated using the formula of $2^{-\Delta\Delta Ct}$ by comparing mRNA levels to the control. Triplicate qRT-PCR analyses were run for each sample.

| Primers | | Sequences (5′→ 3′) | Size (bp) | References |
|---|---|---|---|---|
| β-actin | Forward | TCACCCAACACTGTGCCCATCTACGA | 180 | Mambo et al., 2005 |
| | Reverse | TCGGTGAGGATCTTCATGAGGTA | | |
| hOGG1 | Forward | GCGACTGCTGCGACAAGAC | 250 | Chevillard et al., 1998 |
| | Reverse | TCGGGCACTGGCACTCAC | | |
| APE1 | Forward | GAG TAA GAC GGC CGC AAA GAA AAA | 296 | Collins et al., 2003 |
| | Reverse | CCG AAG GAG CTG ACC AGT ATT GAT | | |
| PARP1 | Forward | CAA CTT TGC TGG GAT CCT GT | 185 | Mayer et al., 2002 |
| | Reverse | TGT TTC CAA GGG CAA CTT CT | | |
| XRCC1 | Forward | CGC TGG GGA GCA AGA CTA TG | 517 | Noe et al., 2004 |
| | Reverse | CAA ATC CAA CTT CCT CTT CC′ | | |

Table 1. Primers of DNA repair enzyme genes and reference gene for RT-PCR analysis.

### 2.5 Statistical analysis

Each experiment was performed three times independently. Statistical evaluations were performed with GraphPad Prism (GraphPad software, San Diego, CA). One-way analysis of variance (ANOVA) was used to determine the significance of the experimental variables.

Mean values for each treatment were compared with the Dunnett's multiple comparison post-test at a 95% confidence interval. Student's t-test was used to compare effects of the temperature.

## 3. Results

Caco-2 cells were treated with sublethal concentrations (100 μM) of hydrogen peroxide at two different temperatures. This concentration of hydrogen peroxide showed no significant effects on the viability of Caco-2 cells under these experimental conditions and was confirmed to generate significant DNA damage including DNA SSBs and oxidative DNA adducts (Min & Ebeler, 2009). Oxidant treatment at 4°C for 10 min has been adopted in several previous studies to reduce DNA repair and so was adopted in this study as a comparison treatment where DNA repair should be minimized (Astley et al., 2002; Collins et al., 1995). The higher temperature (37°C) was used to represent physiological temperature conditions. Neither condition affected cell viability in the present study (data not shown). Levels of DNA damage (SSBs) was monitored over time by comet assay and mRNA levels of several BER enzymes were correspondingly determined by real time qRT-PCR.
Fig. 1 shows DNA damage levels during the recovery time following oxidant treatment at 4°C. Immediately after hydrogen peroxide treatment (time 0), DNA damage increased significantly. Levels of damage then decreased consistently throughout the recovery period. Fig. 2 shows corresponding levels of mRNA for several DNA BER enzymes following oxidant challenge at 4°C. Except for XRCC1, mRNA levels for all DNA repair enzymes varied significantly during the recovery time following oxidant treatment ($p < 0.05$). mRNA levels of PARP1 increased immediately after oxidant treatment at time 0. In contrast, expression levels of hOGG1, which cleaves the 8-oxoguanine lesion, decreased initially (0 h) and remained lower than control throughout the recovery period. APE1 expression also decreased initially (0 h) but then increased again over the recovery period following treatment at 4°C.

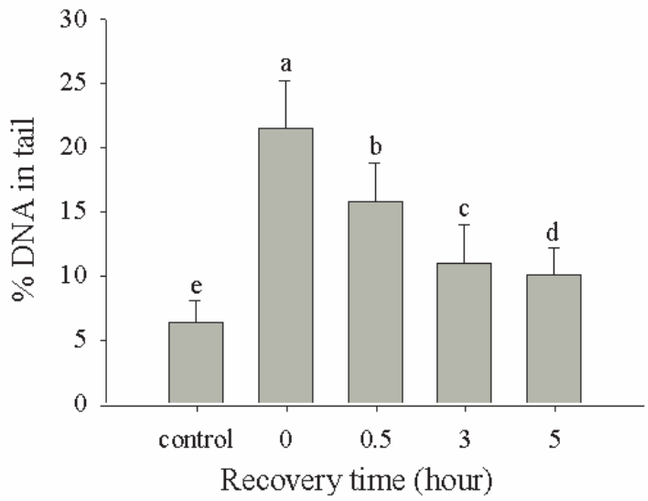



Fig. 1. DNA damage under oxidant challenge at 4°C. Bars represent mean ± SD. Bars not sharing a letter are significantly different ($P < 0.05$) (for each time, n=6 independent replications x 2 slides per replication).

Fig. 3 and 4 show DNA damage change and corresponding mRNA levels of DNA repair enzymes following oxidant challenge at 37°C. Levels of DNA damage increased significantly immediately following hydrogen peroxide treatment although the amount of damage was less than that of cells treated with oxidant at 4°C (t-test; $p < 0.001$) (Fig.3). The level of DNA damage decreased by 39% during the first 0.5 h, a rate of decrease that was faster than that observed following oxidant treatment at 4°C. However, the level of DNA damage did not maintain this rapid decrease over an extended period and DNA damage actually increased slightly after 3 h. Nonetheless, the level of damage was still lower than that at 0 h and levels of damaged DNA again decreased at 5 h.

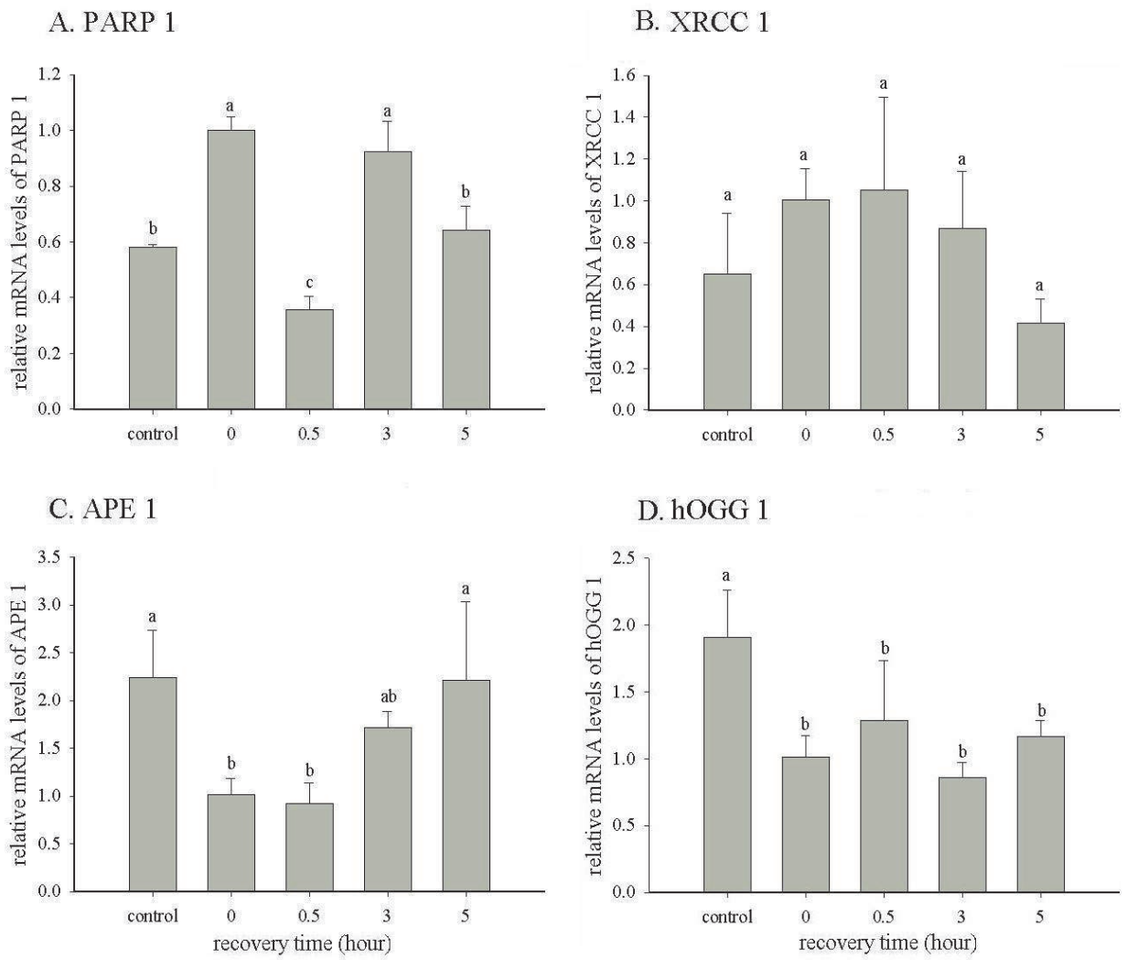



Fig. 2. Relative mRNA levels of DNA repair enzymes under oxidant challenge at 4°C. Levels are reported relative to 0 time. Bars represent mean ± SD. Bars not sharing a letter are significantly different ($P < 0.05$) (for each time, n=3 independent replications and qRT-PCR was analyzed in triplicate for each sample).

Unlike at 4°C mRNA levels did not change significantly for any of the repair enzymes immediately (0 h) after hydrogen peroxide treatment at 37°C (Fig. 4). However, hOGG1 levels did increase at 0.5 h and then gradually decreased during the recovery. XRCC1 levels

also decreased late in the recovery period. On the other hand, APE1 expression increased late in the recovery time (3-5 h) and showed an approximately inverse relationship with hOGG1 levels. mRNA expression levels of PARP1, a SSB recognizing enzyme, changed dynamically at 4°C however, at 37°C PARP1 levels generally were maintained at the basal levels except for decreases which occurred at 3 and 5 h (Fig. 4).

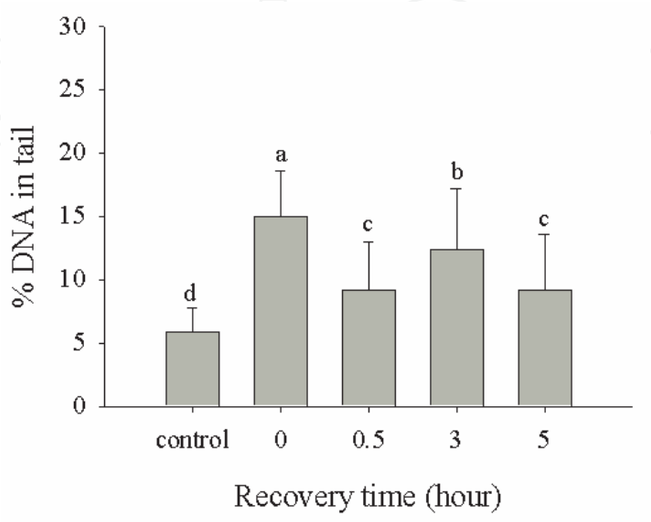



Fig. 3. DNA damage under oxidant challenge at 37°C. Bars represent mean ± SD. Bars not sharing a letter are significantly different ($P < 0.05$) (for each time, n=6 independent replications x 2 slides per replication).

## 4. Discussion

SSBs are a common type of DNA damage produced not only by ROS directly, but also indirectly during DNA repair processes. Moreover, no matter what the origins of SSBs are, SSBs are mostly repaired by the BER pathway. In this study, acute oxidative stress was induced in the Caco-2 cells by $H_2O_2$ treatment. Hydrogen peroxide has been shown previously to generate significant DNA damage including DNA base lesions and SSBs (Cantoni et al., 1987; Dizdaroglu, 1994; Min & Ebeler, 2009). In order to obtain a more complete picture of the cellular response to DNA damaging agents, DNA damage as a function of SSBs and corresponding mRNA expression levels of DNA repair enzymes were monitored. The measured DNA damage is the result of a balance between production of breaks by specific DNA base lesion glycosylases and the sealing of gaps by polymerases and ligases (Cantoni et al., 1987). Among DNA BER enzymes, PARP1, which recognizes and binds to AP sites, and XRCC1, a coordinating protein of the DNA BER pathway, were evaluated here. In addition, hOGG1, a glycosylase which cleaves 8-oxoguanine, and APE1, an endonuclease which cleaves AP sites, were also monitored. We examined DNA damage and repair at two different temperatures. Several studies have induced DNA damage at low temperature in order to minimize the possibilities of DNA repair (Astley et al., 2002; Collins et al., 1995). Accordingly our study adopted low temperature as a reference condition and also monitored responses at 37°C in order to elucidate the biochemical responses to DNA damaging agents at physiological temperature.

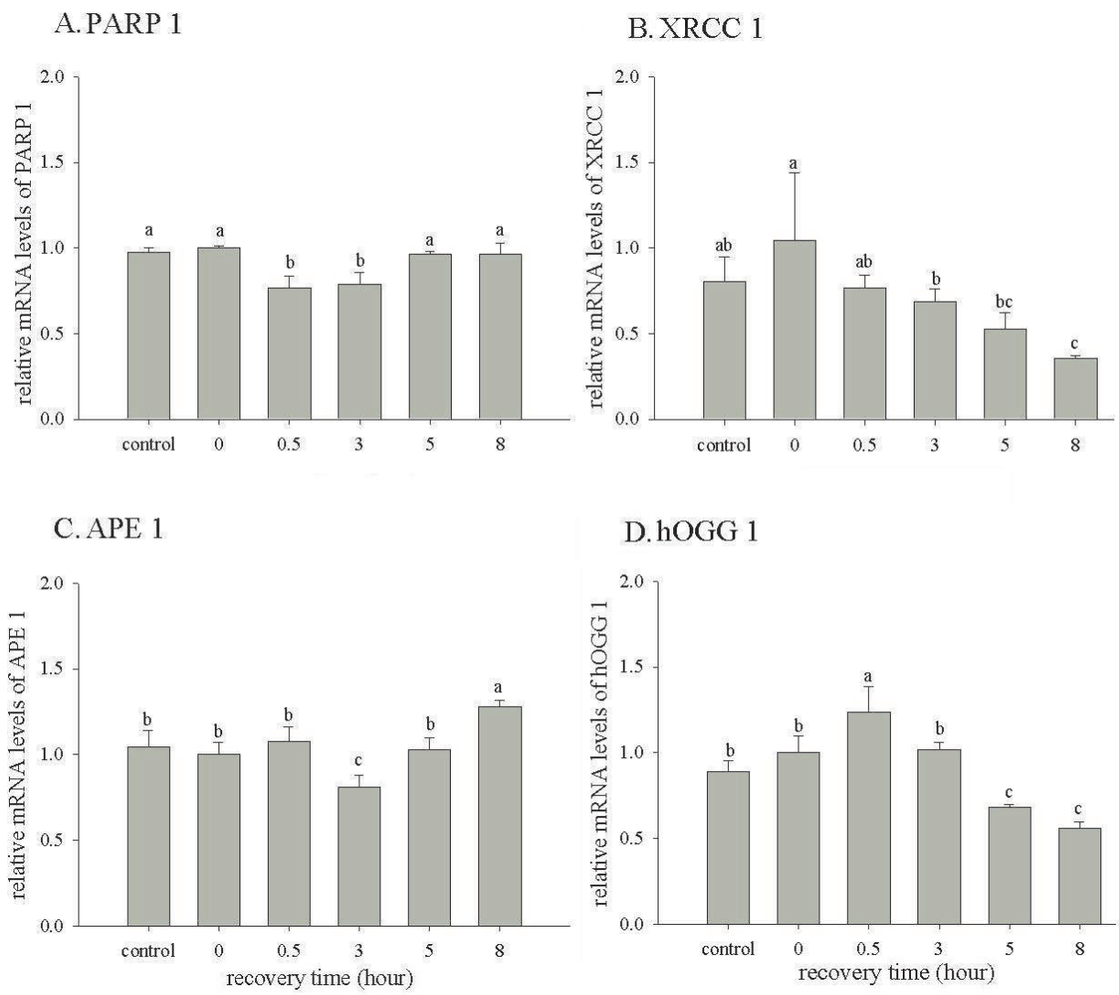



Fig. 4. Relative mRNA levels of DNA repair enzymes under oxidant challenge at 37°C. Levels are reported relative to the 0 time. Bars represent mean ± SD. Bars not sharing a letter are significantly different ($P < 0.05$) (for each time, n=3 independent treatments and qRT-PCR was analyzed in triplicate for each sample).

Higher levels of DNA damage followed by steady repair over time were observed following oxidant treatment at 4°C compared to treatment at 37°C. Foray et al. (1995) have shown more DNA double strand breaks at 4°C compared to 37°C following ionizing radiation, consistent with our study using a chemical oxidant. In our study, mRNA levels of PARP1 responded quickly to increased DNA damage at 4°C while the expression levels of hOGG1 and APE1 decreased immediately following oxidant treatment. Early decreases of APE 1 have been shown by Morita-Fujimura et al. after cold-injury induced brain trauma in mice (Morita-Fujimura et al., 1999). The increase of PARP1 immediately after oxidant treatment and the late gradual increase of APE1 may be factors contributing to the decrease in DNA damage (i.e., increase in repair) over time following the low temperature oxidant challenge. Therefore, although low temperature is typically associated with reduced metabolic and

enzymatic activity, the effects of cold temperature on DNA damage appear to be complex and more investigations are needed to understand the effects of cold temperature on DNA damage and subsequent repair activity.

Levels of SSBs initially and over time, were different following oxidant challenge at 37°C compared to oxidant challenge at 4°C. It is possible that DNA repair was actually initiated during the hydrogen peroxide treatment at the higher temperature resulting in lower DNA damage levels immediately after oxidant treatment. This could at least partially account for the lower level of damage observed at 0 h compared to the treatment at 4°C. In addition, mRNA expression patterns of the repair genes were different from those at 4°C. For example, increased levels of hOGG1 within the first 0.5 h following oxidant challenge were consistent with greater DNA repair (i.e., decreased levels of DNA damage) that was observed following oxidant treatment at 37°C. The inverse relationship between APE1 and hOGG1 mRNA levels from 3 to 8 h at 37°C is consistent with hOGG1 producing AP sites which then induce mRNA production of APE1 (Hill et al., 2001).

Measuring the mRNA expression levels of DNA BER genes has been indicated to be a sensitive end point for determining the effects of chronic oxidative stress to DNA (Rusyn et al., 2004). In addition, consistent with our results following oxidant challenge at 37°C expression of hOGG1 mRNA, has been shown to be inducible responding to various conditions and reflecting DNA repair, at least initially. However, our results indicate that no single gene reflects the overall DNA repair response at any one point in time following oxidant treatment and it is difficult to fully relate changes in mRNA expression levels to the observed DNA damage repair kinetics.

## 5. Conclusions

Our study indicates that DNA damage induced by oxidant at physiological temperature (37°C) is lower as compared to damage at low temperatures. In addition, the pattern of mRNA expression of DNA repair processing enzymes is different over time following treatment. Some of the changes in DNA damage levels over the extended recovery period could be associated with the overall pattern of mRNA expression of several DNA repair enzymes, however, our results indicate that an individual gene alone may not accurately reflect the overall DNA repair capacity. Our study also indicates that protocols using low temperatures to minimize DNA repair may actually result in conditions that enhance DNA damage and result in very different repair kinetics than those that occur at a physiologic temperature. Experimental protocols should be carefully evaluated and interpreted if nonphysiologic conditions are used. Further studies comparing oxidative damage, mRNA expression and protein/enzyme levels and their activity are needed in order to fully understand the molecular responses to DNA damaging agents in the DNA damage/repair processes under variety of conditions.

## 6. Acknowledgments

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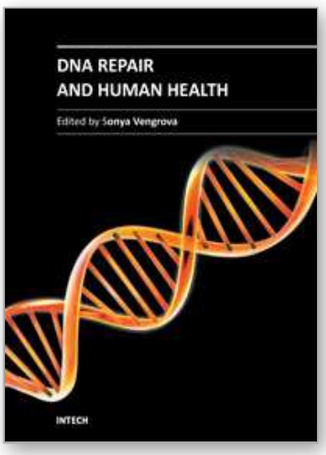
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**DNA Repair and Human Health**

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Over the past decades, great advances have been made in understanding the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research of the mechanisms of DNA repair is merging with clinical research, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach enables understanding of the disease mechanisms and is invaluable in improving diagnostics and prevention, as well as designing better therapies. This book highlights the central role of DNA repair in human health and well-being. The reviews presented here, contain detailed descriptions of DNA repair pathways, as well as analysis of a large body of evidence addressing links between DNA damage repair and human health. They will be of interest to a broad audience, from molecular biologists working on DNA repair in any model system, to medical researchers.

**How to reference**

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