Changes in expression profiles of genes associated with DNA repair following induction of DNA damage in larval zebrafish *Danio rerio*

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DNA repair is initiated by transcription of genes in response to specific types of damage. Breaks in DNA strands (single and double) are repaired predominantly through non-homologous end-joining (NHEJ) or homologous recombination (HR), but progression of repair and changes in expression profiles of genes involved are unknown. DNA damage was induced in zebrafish larvae by brief exposure (10 min) to hydrogen peroxide (H₂O₂; 100 mM), and induction of DNA strand breaks was assessed by single-cell gel electrophoresis (comet) assay over 24 h. H₂O₂ was selected because it is eliminated rapidly after induction of DNA damage. DNA damage [mean ± standard error of the mean (SEM), % tail DNA] increased significantly immediately after 10-min H₂O₂ exposure (35.4 ± 3.8; control 17.2 ± 2.0), but damage did not differ from control levels 24 h after exposure (9.2 ± 0.4; control 9.9 ± 0.9). At 0-, 1-, 3-, 6-, 12- and 24-h post-exposure, quantitative reverse transcriptase–PCR was conducted to assess expression of selected genes involved in DNA repair including *xrc5*, *xrc6* (NHEJ), *rad51* (HR) and *gadd45a* (DNA damage detection). Expression (maximum fold-change ± SEM, triplicate samples of 40 larvae) of each gene increased rapidly (within 6 h) after exposure to 100 mM of H₂O₂: 1.8 ± 0.2, *rad51*; 1.7 ± 0.2, *xrc5* and 1.5 ± 0.1, *xrc6*. Acute exposure (200 mM of H₂O₂) caused 10% larval mortality within 2 h, upregulated *gadd45a* (5.0 ± 0.8), but did not change expression of *rad51*, *xrc5* or *xrc6*. Expression profiles (critical exponential model) were similar among genes but differed relative to time and among independent experiments. Results indicate that repair mechanisms are initiated rapidly after DNA damage, that gene expression profiles vary according to potency of H₂O₂ exposure and that examination of the time course of gene expression changes is necessary to understand the complete gene response over time.

Introduction

Endogenous and exogenous agents cause DNA strand breaks that must be repaired to preserve cellular function. A strand break can occur in one or in both strands, and depending on the type of breakage, different repair mechanisms will be induced to resolve the DNA damage and reduce breaks within damaged cells (1,2). Once damage to DNA (e.g. strand breaks) is detected, the cell cycle is arrested and repair is initiated (3). A series of proteins mediate damage recognition, signalling, end processing and end re-joining, via either non-homologous end-joining (NHEJ) or homologous recombination (HR) pathways (4). When the homologous sister ends of the broken DNA strands are in close proximity, repair using the intact sister template can be straightforward and accurate through the HR pathway (5). Rad proteins bind to ends of broken DNA strands, spanning the gap and recruiting ligating enzymes to re-join the strands (4,8,9).

Repair of DNA damage is initiated by induction of genes that code for products that drive the repair process. Key genes involved in NHEJ are *xrcc5* and *xrcc6* (encoding for Ku80 and Ku70 protein, respectively), and *rad51* is critical for HR repair (10). These genes involved in NHEJ and HR repair are induced after radiation (11,12) and metal (13,14) exposure, in response to induction of strand breaks. *In vitro* studies reported induction of *xrcc5* 12 h after exposure to UV-B (12) and 24 h after exposure to copper (14). In adult zebrafish, a 10-fold induction of *rad51* expression was reported in liver after 63-day dietary exposure to 13.5 μg/g methylmercury (15), a 32-fold induction in expression after 7-day aqueous exposure to 9.6 ± 2.9 μg/l cadmium (13), a 5-fold peak in expression in gills after 28-day aqueous exposure to 130 ± 34 μg/l copper followed by 8-day depuration (16) and a 2-fold increase in testes after 14-day aqueous exposure to 25 mg/l cobalt (17). It has been suggested that single-strand breaks are induced and repaired rapidly compared with more complex double-strand breaks (18), but the timing of induction of damage and expression of these repair genes has not been established.

Understanding the timing of gene expression is important for interpreting the dynamics of gene regulation (19). Frequently, gene expression studies have not collected samples at enough time points to permit effective assessment of the change in gene expression over time (13,20) and this can lead to comparisons in expression of particular genes at time points that do not necessarily reflect important moments in the expression profile of the genes. Advances have been made in modelling large microarray data sets of gene expression changes among treatments and over time, but a focused comparison of the timings of expression in genes is uncommon (21), and this approach has not been applied to DNA repair genes. Evaluation of time-related expression profiles of individual genes has been approached by a critical exponential curve model, which was reported to be an improvement over other models including analysis of variance (ANOVA), clustering or network models (21). The critical exponential curve model enables time and level of expression to be compared for
individual genes across experimental treatments (21), and this approach could be useful for investigating changes in expression of DNA repair genes.

The objectives of this study were to induce DNA strand breaks in larval zebrafish and to evaluate changes in expression of key genes involved in DNA repair during the repair period (24h). Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was selected as the agent to induce DNA damage because it is highly reactive and does not persist after fish are transferred to clean water, and it has been used as a reference genotoxicant in zebrafish embryo (22) and larvae (17,20). Expression profiles of \textit{xrcc5}, \textit{xrcc6}, \textit{rad51} and \textit{gadd45a} (a gene involved in detection of DNA damage) were evaluated in three separate \textit{in vivo} time-course experiments, and the time course of expression was modelled by a critical exponential model (21).

Materials and methods

Larvae

Wild-type adult zebrafish (\textit{Danio rerio}, 4–5 months old) were reared in re-circulating aquaria in the Zebrafish Research Facility at Plymouth University, UK, maintained under routine approved animal welfare protocols. Water quality parameters were measured daily (mean ± SD) for temperature (26 ± 1°C), pH (6.7 ± 0.3) and dissolved oxygen (92 ± 3%), and ammonium, nitrate and nitrite levels were analysed weekly (<0.02, <20 and <0.1 mg/l, respectively). Photoperiod was 12:12 h light and dark cycle, and fish were fed three times daily with live brine shrimp nauplii. \textit{Artemia} species or dry fish flake mix (equal proportions of \textit{ZM} Systems flakes, brine shrimp, spirulina and TetraMin® stable flake). Stock fish were routinely bulk spawned, and eggs were collected and reared in 50 ml Petri dishes with daily water changes. For all larval exposures, 72-h post-fertilisation (hpf) hatched larvae were selected.

Validation of single-cell gel electrophoresis (comet) assay

Unless otherwise specified, all chemicals were sourced from Sigma-Aldrich (Dorset, UK). Zebrafish larvae (30 newly hatched, 72 hpf) were morphologically homogenised (pellet pestle; Sigma) in 100 μl of Dulbecco’s phosphate-buffered saline (Gibco), and 5-μl aliquots of cell suspension were incubated for 10 min with H\textsubscript{2}O\textsubscript{2} (nominally 0, 1, 10, 25, 50, 100, 200 and 500 μM; concentration range based on previous study) (22), before centrifugation (8000g) to pellet the cells. The supernatant was removed, and the cell pellet was washed and re-suspended in 10 μl of Dulbecco’s phosphate-buffered saline. The re-suspended cells (10 μl) were mixed with 200 μl of low-melt-point agarose, and DNA damage was assessed by alkaline single-cell gel electrophoresis (comet) assay. Comet assay was carried out following the routine procedures (22), with some modifications for somatic cells. In brief, 10 μl of re-suspended cells were mixed with 180 μl of low-melt-point agarose (0.5%), dropped onto a slide pre-coated with 1.5% normal melting point agarose (dried overnight) and flat-topped with coverslips. Gels were left to set for 1 h at 5°C before removal of coverslips, and then slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 M Tris, 1% Triton X-100, 100 mM DMSO, 34 mM s-sodium saccharose, pH 10) for 1 h. After lysis, slides were placed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min of unwinding, followed by electrophoresis (25 V, 280–350 mA) for 20 min. Slides were washed in neutralising buffer (0.4 M Tris–HCl, pH 7.5) and distilled water, allowed to dry overnight and scored for % tail DNA (Leica DMFR microscope, 100 cells per slide; Komet, Kinetic Imaging, Nottingham, UK) following routine procedures (23,24).

In vivo exposures of larvae to H\textsubscript{2}O\textsubscript{2}

Due to the highly reactive nature of H\textsubscript{2}O\textsubscript{2}, exposures were carried out in tripli-
cate to ensure triplicate treatments and triplicate controls were time matched, and each individual experiment used the same H\textsubscript{2}O\textsubscript{2} stock for each treatment. Newly hatched larvae (72 hpf) were counted into 50-ml plastic dishes (9 cm diameter, 40 larvae per dish, 3 dishes per treatment) in clean ‘fish water’ (aquarium system water). For each concentration, H\textsubscript{2}O\textsubscript{2} was added to the dishes (total volume 40 ml) and the larvae were exposed for 10 min (20). Control dishes contained 40 ml of ‘fish water.’ After 10-min exposure to H\textsubscript{2}O\textsubscript{2}, larvae were poured through a sieve and returned to clean ‘fish water’ to start the recovery period. Control dishes were also washed through sieve and larvae returned to clean ‘fish water.’

An initial experiment was conducted to establish the response of larvae exposed to different concentrations of H\textsubscript{2}O\textsubscript{2} (nominally 0, 50, 100, and 200 mM H\textsubscript{2}O\textsubscript{2}), followed by 0 and 24 h of recovery in clean water. Larvae were collected and sampled for comet assay as outlined in Validation of single-cell gel electrophoresis (comet) assay section [with the exception of mechanical homogenisation in lysis buffer (RLT buffer, Qiagen RNNeasy MiniKit), three slides analysed per sample]. Toxicity was assessed by mortality after 24-h recovery.

Three independent recovery time-course experiments were conducted to investigate gene expression responses under different experimental conditions. In Experiment 1, larvae were exposed to a nominal concentration of 100 mM H\textsubscript{2}O\textsubscript{2} for 10 min, followed by a recovery period of 0, 6, 12 and 24 h in clean water. Comet assay was conducted to assess DNA damage at 0- and 24-h recovery time points. In Experiment 2, larvae were exposed to a nominal concentration of 100 mM H\textsubscript{2}O\textsubscript{2} for 10 min, followed by a shorter recovery time course of 0, 1, 3 and 6 h in clean water. In Experiment 3, larvae were exposed to an acute nominal concentration of 200 mM H\textsubscript{2}O\textsubscript{2} for 10 min, followed by a recovery time course of 0, 0.25, 0.5, 1, 2 and 2.5 h in clean water. Larvae from all recovery time points were sampled for gene expression analysis. Samples (triplicate exposed and triplicate controls at each time point) were mechanically homogenised in lysis buffer (RLT buffer, Qiagen RNNeasy MiniKit) and frozen at −80°C for gene expression analysis.

RNA extraction and complementary DNA synthesis

Total RNA was extracted (RNNeasy MiniKit for animal tissue; Qiagen) from samples of 30 larvae, mechanically homogenised in RLT buffer and frozen at −80°C, following manufacturer’s protocol with initial sonication (3–5 s). Additional steps included further tissue break-up (QiShredder column; Qiagen) and a 15-min DNase treatment. RNA was eluted into 30 μl, and concentration and quality of total RNA was determined by spectrophotometer (NanoDrop, ND-1000 Spectrophotometer). All samples were diluted to 100 ng/μl of total RNA, and 800 ng was used to synthesise complementary DNA (cDNA) following the manufacturer’s protocol (iScript cDNA Synthesis Kit, BioRad). The cDNA was stored at −80°C until quantitative reverse transcriptase–PCR (qRT–PCR) gene expression analysis.

Quantitative reverse transcriptase–PCR

Primers were previously designed, selected and verified (17), and details are listed in Table I. Lyophilised primers (Eurofins MWG Operon, Ebersberg, Germany) were reconstituted to 100 μmol with RNase-free water and mixed with SYBR Green JumpStart Taq ReadyMix (BioRad) to give final reaction concentration of 375 nM in a 20 μl of total volume. Fluorescence was detected (StepOne Real-Time PCR System; Applied Biosystems) over 40 cycles, cycling conditions were 10 s for denaturing, primer-specific annealing at 55–60°C (see Table I) and extension at 72°C. For analysis, the cycle threshold was set to 25 000 for all qRT–PCR runs. A standard curve of cDNA template (pooled template from each

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<th>Gene</th>
<th>Reference sequence number</th>
<th>Forward (5′–3′)</th>
<th>Reverse (5′–3′)</th>
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<th>Annealing temperature (°C)</th>
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<td>TACATCCCTGGATGTGGTCCGT</td>
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sample within experiment) was run on each plate to allow for within-experiment plate normalisation.

Data analyses
Statistical analyses were conducted with STATGRAPHICS 5.1 (Statistical Graphics Corp.). Comet assay data (% tail DNA) were arcsine transformed before a general linear model (GLM) was applied to test for significant dose response following both in vitro and in vivo exposures. For gene expression analysis, the efficiency of qRT-PCR was calculated (e = \(10^{\text{Ct}}\text{mM}^{-1}\)) from the standard curve for each plate. Samples from the same experiment run over multiple plates were adjusted to the plate with the efficiency closest to 1 by resolving for slope and intercept of the standard curves. Only efficiencies between 0.9–1.1 were accepted for further analysis, and comparative quantification (2^\(-\Delta\Delta\text{Ct}\)) was used for calculating fold-changes in the gene of interest normalised to \(\beta\)-actin (ΔCt) calculated by mean \(\beta\)-actin C for 0-, 6- and 12-h time points, and mean C for 24-h time point and to time point controls (ΔΔCt) (25). The kinetic response in gene fold-change over time was modelled by a critical exponential curve \(y(x) = A + (B + Cx)^R\), where A, B, C and R are model parameters. y is the fold-change gene expression response and x is time, as proposed for time-course analysis of gene expression (21). The model was fitted using SigmaPlot (Systat Software Inc.). Within-time point differences in gene expression between control and exposed samples were tested by one-way ANOVA.

Results
No changes in behaviour of larvae were observed upon addition of \(\text{H}_2\text{O}_2\) to the fish water. After 10-min exposure to \(\text{H}_2\text{O}_2\) and rinsing in clean fish water, larvae initially maintained their position motionless on the bottom of the container before resuming normal swimming behaviour. No larvae died 24 h after the 10-min exposure to nominal concentrations of 50 mM \(\text{H}_2\text{O}_2\) and, below, and mortality was 27.5 and 23.1% at nominal concentration of 100 and 200 mM, respectively (Figure 1). DNA strand breaks were induced by exposure to \(\text{H}_2\text{O}_2\) in both larval tissue homogenates (in vitro) and in exposed larvae (in vivo). Control levels of DNA damage in larval tissue homogenates (in vitro, no exposure to \(\text{H}_2\text{O}_2\)) were 26.6 ± 2.1 standard error of the mean (SEM), % tail DNA, and damage increased significantly to 70.8 ± 2.1 SEM, % tail DNA after in vitro exposure to a nominal concentration of 500 µM \(\text{H}_2\text{O}_2\) (Figure 2). In comparison, control levels of DNA damage in larvae exposed in vivo was 8.3 ± 3.4 SEM, % tail DNA, and larvae exposed to a nominal concentration of 200 mM \(\text{H}_2\text{O}_2\) had levels of DNA damage up to 36 ± 1.2 SEM, % tail DNA (Figure 1). Larvae exposed to \(\text{H}_2\text{O}_2\) in vivo had significantly lower levels of DNA damage 24 h after return to clean water, and damage did not differ significantly from controls (% tail DNA: 8.6 ± 0.8 SEM, nominal concentration of 200 mM \(\text{H}_2\text{O}_2\)); unexposed control larvae, 11.1 ± 1.6 SEM, % tail DNA; Figure 1). In Experiment 1 (recovery time course), levels of DNA damage were 35.4 ± 3.8% tail DNA in exposed larvae (unexposed controls: 17.2 ± 2.0 SEM, % tail DNA), and 24-h recovery in clean water indicated no significant difference in DNA damage between \(\text{H}_2\text{O}_2\) exposed and control larvae (exposed: 9.2 ± 0.4 SEM; unexposed controls: 9.9 ± 0.9 SEM, % tail DNA).

The concentration and potency of \(\text{H}_2\text{O}_2\) differed among the three recovery time-course experiments (nominal concentration 100 mM \(\text{H}_2\text{O}_2\) in Experiments 1 and 2, and nominal concentration of 200 mM \(\text{H}_2\text{O}_2\) in Experiment 3). No larvae died in Experiments 1 and 2, and larval mortality reached 10.0% by 2 h after exposure in Experiment 3. Induction of gadd45a transcription (gene coding for detection of DNA damage) was rapidly (<0.5 h) induced to 5.0 ± 0.9 (mean ± SEM, n = 3) and remained elevated through 2.5 h after exposure in Experiment 3 (Figure 3). Although the nominal \(\text{H}_2\text{O}_2\) exposure of 100 mM was the same in Experiments 1 and 2, the gadd45 induction profile over time differed between these experiments. In Experiment 1, the induction of gadd45a persisted longer than 12-h post-exposure, whereas expression returned to control levels within 3 h of exposure in Experiment 2.

There was no statistically significant difference in mean C values for \(\beta\)-actin between exposed and control larvae at each time point evaluated; therefore, use of \(\beta\)-actin as a housekeeping gene for normalisation was justified. There was a difference (GLM, \(P < 0.05\)) in expression of \(\beta\)-actin over time, and larvae sampled between 0–12 h post-exposure had lower mean C (17.2 ± 0.1 SEM, n = 18) compared with larvae sampled at 24-h post-exposure (17.9 ± 0.2 SEM, n = 6).

![Fig. 1. DNA damage (% tail DNA, comet assay) in larvae exposed in vivo to increasing concentrations of \(\text{H}_2\text{O}_2\). Dark grey bars indicate larvae sampled immediately after 10-min incubation with \(\text{H}_2\text{O}_2\), significant increase in damage with increasing concentration (arcsine transformed, GLM, \(P < 0.05\)). Light grey bars indicate larvae exposed for 10 min and allowed to recover in clean water for 24 h. Open triangles show increasing mortality with increasing concentration after 24 h. Bar data are means of 3 replicate slides per concentration (technical replicates), 30 larvae sampled and pooled per concentration (except controls with 3 dishes of 30 larvae, three slides per dish, mean ± SEM of three replicate dishes). Mortality data (% dead) after 24 h, n = 40 larvae per concentration (controls in triplicate).](https://academic.oup.com/mutage/article-abstract/28/5/601/1269384/603)
Expression profiles for DNA repair genes *xrcc5*, *xrcc6* and *rad51* differed among the three experiments. Although the level of induction of *xrcc* genes was similar between Experiments 1 and 2, the timing of induction differed (Figure 4). Expression of the *xrcc* genes was upregulated at 6-h post-exposure in Experiment 1, but in Experiment 2, induction occurred at 1 h after exposure and expression was lower than control levels at 6-h post-exposure. The acute 200 mM exposure to H$_2$O$_2$ (Experiment 3) did not alter expression of *xrcc* genes. Modelled gene expression profiles of both the *xrcc* genes indicated early onset of gene induction in Experiments 1 (before 6 h) and 2 (within 1 h), and modelled peak expression of *xrcc5* was earlier than modelled peak expression of *xrcc6*. After the peak in expression of *xrcc5* and *xrcc6*, expression declined, but the timing of the reduction in level of gene expression (return to control levels) was different between Experiments 1 and 2. For both *xrcc5* and *xrcc6*, expression returned to control levels after 12-h recovery in Experiment 1 and after 3 h in Experiment 2. The gene response profiles were similar, but expression profiles differed relative to time.

Fig. 2. Concentration-dependent increase in DNA damage (% tail DNA, comet assay) with exposure to H$_2$O$_2$ in mechanically homogenised larvae (72 hpf) exposed *in vitro* for 10 min (arcsine transformed, GLM, $P < 0.05$). Data are means ± SEM, $n = 100$ nucleoids within exposed sample.

Fig. 3. Relative fold-change ($2^{-\Delta\Delta C_t}$) of *gadd45a* in zebrafish larvae exposed to nominal concentrations of 100 mM H$_2$O$_2$ (Experiment 1: white diamonds; Experiment 2: grey circles) or 200 mM H$_2$O$_2$ (Experiment 3: black squares) for 10 min, followed by a period of recovery in clean water, $n = 40$ larvae per dish, error bars are SEM from $n = 3$ dishes per recovery time point. Dotted line indicates no induction in control samples, $n = 3$ dishes per recovery time point. Curves indicate fitting of critical exponential curve, $y(x) = A + (B + Cx) e^{-Rx}$, where A, B, C and R are model parameters, $y$ is the fold-change in gene expression response and $x$ is time [adapted from Eastwood et al. (21)]. Asterisks indicate within-time point significant difference from control, $P < 0.05$, one-way ANOVA.
Expression profiles of DNA repair genes

Expression of \textit{rad51} was upregulated (maximum fold-change $1.80 \pm 0.28$, mean $\pm$ SEM, $n = 3$) after 6-h recovery in Experiment 1 (Figure 4A). In Experiment 2, \textit{rad51} was induced after 1-h recovery (maximum fold change $1.37 \pm 0.3$, mean $\pm$ SEM, $n = 3$), and after 3 h, there was no induction and expression had returned to control levels (Figure 4A). Modelled expression profiles of \textit{rad51} differed among experiments with respect to time, but the gene responses were similar. The acute exposure in Experiment 3 resulted in no upregulation in \textit{rad51}.

Based on the critical exponential model, the timings of the highest level of gene induction for Experiment 1 were 2.67, 3.00, 3.22 and 5.03 h for \textit{xrcc5}, \textit{xrcc6}, \textit{rad51} and \textit{gadd45a}, respectively. The modelled profiles from Experiment 2 indicated highest expression at 0.49, 0.74, 0.98 and 0.51 h for \textit{xrcc5}, \textit{xrcc6}, \textit{rad51} and \textit{gadd45a}, respectively. A comparison between the model parameters (Table II) suggested that \textit{xrcc5} and \textit{xrcc6} had similar and closely matched peak expression profiles from Experiment 1 but were dissimilar at earlier periods of recovery in Experiment 2.

**Discussion**

\textit{In vivo} exposure of zebrafish larvae to nominal concentrations of 100 and 200 mM H$_2$O$_2$ caused mortality and damage to DNA. H$_2$O$_2$ was selected as the reference genotoxicant because it efficiently induces DNA strand breaks through production of reactive oxygen species (26) and is rapidly neutralised without residual persistence in the organism (i.e. no residual effects) (27,28). Although there are concerns regarding heterogeneity of cell response after H$_2$O$_2$ exposure (29,30), H$_2$O$_2$ is commonly used as a reference toxicant for genotoxicity studies (including zebrafish) (20,22) and is particularly suited for time-course experiments (27).
reactivity of $\text{H}_2\text{O}_2$, complicates inter-experiment comparisons of nominal concentration responses because $\text{H}_2\text{O}_2$ of the same nominal concentration may not be of similar potency among different experiments. The difference in mortality among exposures of the same nominal concentration among experiments in this study indicates that the exposures were of different potency. Control levels of DNA damage in larvae exposed in vitro were consistent with previous in vitro studies with zebrafish sperm (17,22); however, they were higher than control levels in larvae exposed in vivo in this study, suggesting possible increased DNA damage from mechanical tissue homogenisation before in vitro exposure. In vivo exposure in this study resulted in levels of DNA strand breaks consistent with levels detected in zebrafish larvae exposed to 1% $\text{H}_2\text{O}_2$ (presumed equivalence of 8.8 mM, 7–8% tail DNA) in a previous study (20). Exposure of larvae to the concentrations of $\text{H}_2\text{O}_2$ in this study is likely to have primarily induced single-strand breaks in DNA (31); however, double-strand breaks can form when high levels of accumulated single-strand breaks occur in close proximity (32). The alkaline version of the comet assay used in this study assesses predominantly not only single- but also double-strand breaks and is an indicator of overall DNA strand fragmentation (23).

Induced transcription of genes involved in DNA repair and lower DNA damage in larvae at 24 h compared with immediately after exposure indicated repair of damaged DNA occurred. The three DNA repair genes investigated (xrc5, xrc6 and rad51) were induced up to 2-fold, and this induction was consistent with the relatively low levels of induction observed in other studies (e.g. in vitro exposure to copper resulting in approximately 1.2- and 1.4-fold induction of Ku80/xrc5) (12,14). However, rad51 can be highly induced in adult zebrafish, and a 32-fold change in expression has been reported in liver after 7-day exposure to cadmium (13) and 5-fold change in gills after 8-day exposure to uranium (16). The induction of genes involved in NHEJ and HR suggests that both repair pathways were activated as a consequence of the $\text{H}_2\text{O}_2$ exposure, which agrees with suggestions that DNA repair pathways are not mutually exclusive (7,33). DNA repair genes can be constitutively expressed in cells (11,34) due to the importance of DNA repair on cell survival and have been used as housekeeping genes (35). Therefore, the relative number of transcripts of DNA repair genes within actively dividing cells can be constantly high (36), and subtle relative induction in expression could be indicative of increased levels of DNA repair activity.

The highest level of induction of gadd45a was observed in Experiment 3, and this result was consistent with the higher potency of the acute $\text{H}_2\text{O}_2$ exposure. Gene products of gadd45a include DNA damage-inducible (GADD) proteins that are involved in growth arrest and cell cycle regulation (37), and induction of gadd45 has been reported after exposure to ionising radiation (38) and metals (13,15). Expression of gadd45 was induced 3-fold after 14-day exposure to cobalt (17) and 55-fold after 7-day exposure to cadmium (13); and this high level of induction has led to the suggestion that gadd45 expression could be an indicator of the occurrence of DNA damage (39). If expression of gadd45 is used as an indicator of the level of DNA damage in this study, the most DNA damage occurred in the acute $\text{H}_2\text{O}_2$ exposure (Experiment 3) followed by Experiment 1 and then by Experiment 2. In Experiment 3, some (10%) fish died from the acute $\text{H}_2\text{O}_2$ exposure and DNA repair genes in surviving fish were not induced, which suggested that detoxification systems were overwhelmed and perhaps cell cycle arrest and apoptosis occurred consistent with previous studies (39,40). Although the nominal concentration of $\text{H}_2\text{O}_2$ was the same in Experiments 1 and 2, differences in the potencies of the $\text{H}_2\text{O}_2$ in these exposures were indicated by differences in gadd45a gene expression, which suggests greater DNA damage in Experiment 1 compared to Experiment 2. Evidence for higher $\text{H}_2\text{O}_2$ potency in Experiment 1 is indicated by the prolonged induction of gadd45a up to 6 h recovery in Experiment 1.

The level of induction of DNA repair gene transcription was similar between Experiments 1 and 2, but expression profiles differed likely because of differences in $\text{H}_2\text{O}_2$ potency (although nominal $\text{H}_2\text{O}_2$ concentrations were the same). In Experiment 1, a marginally higher level of induction of the DNA repair genes (greater measured fold-change for all genes except xrc5) was observed, and the induction of genes continued beyond 6 h after exposure. In contrast, induction of DNA repair genes was lower and returned to control levels within 3 h of exposure in Experiment 2. The lack of induction of DNA repair genes in Experiment 3 (200 mM $\text{H}_2\text{O}_2$) was most likely because cells were responding to an overwhelming toxicity response (e.g. cell cycle arrest) rather than regulation of targeted DNA repair mechanisms.

The profile of the gene expression response was similar among all the genes investigated and between different experiments, but they differed with respect to time. Timing of induction is of particular importance for DNA repair-related genes, as the timing of repair genes has been suggested to be critical (in vitro) in triggering alternative pathways to repair.

Table II. Model parameters of fitting a critical exponential curve, $y(x) = A + (B + Cx) R^x$, where $A$, $B$, $C$ and $R$ are model parameters, $y$ is the fold-change ($2^{\Delta \text{ACT}}$)

<table>
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<th>Gene</th>
<th>Experiment number</th>
<th>Recovery time course (h)</th>
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Fitted model used to determine estimated timing of peak in gene expression response and magnitude of estimated response.
such as apoptosis (41). If a single time point is selected for analysis of gene expression without knowledge of the expression profile over time, conclusions made about treatment effects on gene expression may not be valid (19). The expression and time profile of *xrcc5* and *xrcc6* were similar perhaps because they are involved in the same NHEJ repair mechanism (10), and the model indicated the importance of early recovery time periods for induction of these genes. Although further evidence is required to understand the relation between gene expression profiles among genes and how these relate to cellular physiology, models of the gene expression profiles can give some indications from which hypotheses can be drawn for subsequent testing. For example, modelled gene expression profiles of the DNA repair genes in this study could indicate earlier induction of the NHEJ DNA repair pathway, followed by a later response in HR.

The critical exponential model suggested that time-related expression profiles of *xrcc5*, *xrcc6*, *rad51* and *gadd45a* were similar. The model for Experiment 1 indicated that the maximum expression for each gene occurred after the initial measured gene expression time point (0h), but before the measured 6-h post-exposure samples. Experiment 2 confirmed the rapid modelled induction in expression and also suggested the exposure was less potent because, despite rapid induction, gene expression levels dropped rapidly, which is an indication that the gene was rapidly switched on and then off. However, the few measured time points during the recovery makes interpretation of the modelled time profile somewhat speculative. The model was selected because the shapes of the time profiles of the genes selected in this study were similar to other transcription profiles where the model has been applied (21). Applying this model to individual gene expression profiles allows for comparisons in model parameters (Table II) for key genes of interest and enables formation of hypotheses regarding differences in expression profiles between genes and possible timings of gene responses beyond simple comparisons of significant differences at certain times (21). The *xrc* genes modelled in this study had similar values for model parameter A, which dictates the magnitude of response, and model parameter B, which dictates the initiation of response, in both Experiments 1 and 2. Modelled expression of *rad51* differed from the *xrc* genes in model parameters A and B, which suggests that it is functionally distinct despite overall similarity in the shape of the curve (model parameter C). A comparison between fitted model parameters for *gadd45a* and the three DNA repair genes indicate reduced initial amount of *gadd45a* mRNA (negative model parameter B) that suggests a delayed induction of response and functional dissimilarity from the action of DNA repair in these exposures. A limitation in modelling the response of the selected genes in this study is lack of early time point samples to verify early induction of gene response. More resolution in characterising the early induction period (0–6 h post-exposure) indicated that induction of *xrcc5* was earlier than the 1h measured time point. Modelled expression of *rad51* and *xrcc6* from 0–1 h recovery period matched the modelled expression profile from Experiment 1 closely.

Conclusion

H$_2$O$_2$-induced DNA damage in zebrafish larvae is repaired rapidly after increased transcription of various genes involved in DNA repair. Upregulation of *xrcc5*, *xrcc6* and *rad51* is consistent with induction of both NHEJ and HR repair pathways within 6h of recovery. Induction of gene expression was subtle (<2-fold change) and is in agreement with other studies and within the expected context of genes that are constitutively expressed in cells. Results indicate the importance of establishing time-related changes in expression of genes because, if single time points are selected inappropriately, incorrect conclusions could be drawn. For example, in this study, if samples were collected only at 24-h post-exposure, no change in expression of DNA repair genes would have been detected. Modelling profiles of gene expression changes can enable comparisons in the timing of induction among genes and can inform on the possible sequence of induction of gene product function involved in the process of DNA repair. For example, in this study, modelling of time profiles of induction of *xrcc* genes and *rad51* suggests an earlier induction of the NHEJ DNA repair pathway, followed by HR, and it would be of interest to test this hypothesis in experiments looking at the gene products such as DNA repair proteins. In the context of this study, expression profiles of *xrcc* genes were similar (initiation of induction, time of peak induction and magnitude of induction) within experiments after exposure to H$_2$O$_2$ and shared a similar response curve shape with *rad51*, in support of the involvement of both repair pathways (NHEJ and HR) in the overall repair response to damage by H$_2$O$_2$. Differences in H$_2$O$_2$ potency contribute to differences in DNA damage, expression of genes that code for DNA damage detection (i.e. *gadd45*) and the expression profiles of DNA repair genes.

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