Photochemistry and Photobiology, 2009, 85: 220–226

Time-course Expression of DNA Repair-related Genes in Hepatocytes of Zebrafish (*Danio rerio*) After UV-B Exposure

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Received 20 February 2008, accepted 13 June 2008, DOI: 10.1111/j.1751-1097.2008.00422.x

ABSTRACT

The objective of this study was to evaluate the time-course effects of UV-B exposure on expression of genes involved in the DNA repair system of zebrafish (*Danio rerio*) hepatocytes, a highly competent species in terms of damage repair induced by UV radiation. For gene expression analysis (RT-PCR), cells were exposed to 23.3 mJ cm^{-2} UV-B, which was the dose that affected viable cell number (reduction of 30% when compared with the control group) and produced no visual alteration on cell morphology. The early response observed (6 h) showed induction in the expression of the *CDKI* gene (cyclin-dependent kinase inhibitor) and genes related to DNA damage repair (mainly *XPC* and *DDB2*), while the late response observed (24 h) was more related to up-regulation of *p53* and genes involved in cell cycle arrest (*gadd45a*, *cyclinG1*). In all times analyzed, the anti-apoptotic gene *Bcl-2* was down-regulated. Another interesting result observed was the up-regulation of the *Apex*-1 gene after UV-B exposure, which could indicate the induction of oxidative lesions in the DNA molecule. In conclusion, these results demonstrate an activation of the DNA repair system in hepatocytes of zebrafish exposed to UV-B radiation, mainly involving the participation of p53.

INTRODUCTION

Ultraviolet radiation is commonly divided into three categories: UV-A (400–315 nm), UV-B (315–280 nm) and UV-C (280–100 nm). Among these, virtually no UV-C arrives at the surface of the Earth, and it is estimated that 1–10% of UV radiation on the Earth's surface is UV-B and over 90% UV-A (1). This UV radiation has the potential to cause adverse effects on organisms, on both aquatic and terrestrial ecosystems. In this regard, UV-B is known to act mostly through direct interaction with the DNA molecule, leading to the formation of photoproducts like cyclobutadipyrimidines (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). On the other hand, most of the effects caused by UV-A are mediated by photooxidative reactions, which can also cause DNA damage (2–4).

Regardless of the fact that not only UV radiation but also other agents like environmental contaminants and byproducts of normal cellular metabolism can damage the DNA molecule, DNA repair activities are extremely important because unrepaired DNA damage has the potential to be mutagenic, cytotoxic and carcinogenic. Collectively, the response systems to DNA damage that reduce the yield of mutations and chromosomal aberrations are known as DNA repair systems (4). The components of the DNA damage response act in three levels: (1) arresting the cell cycle to allow time for DNA repair; (2) triggering the signal transduction events to activate the repair components; and (3) directly reversing, excising or tolerating DNA damage *via* constitutive and induced activities (5). If DNA damage is not repaired, cells undergo complex enzymatic reactions that might lead to apoptosis, necrosis or other forms of cell death (6,7).

Several components of the DNA repair system are described in the literature. Among these, the tumor suppressor p53 has a central role in the cellular stress responses, including DNA damage induced by UV-B radiation. p53 elicits its normal functions mainly by acting as a transcription factor, regulating the transcription of genes involved in cell cycle arrest (*e.g. p21*, *gadd45*, *cyclinG1*), DNA repair (*e.g. XPC*, *DDB2*) and apoptosis (*e.g. bcl-2*, *bax*) (1,8,9).

In spite of extensive information concerning the expression profile of components of the DNA repair system that indicate a general pattern, many contradictory results are found in the literature. Many genes that are induced after DNA damage in some studies are not altered or are repressed in others (10–12). These discrepancies may be due to the type of cellular injury, the dose/concentration of the used agent, the time after damage that the response was analyzed, the organism/organ/cell differences and other factors. According to Begley and Samson (5) some factors like the stage of the cell cycle (resting or cycling), the position of the lesion, protein abundance and kinetic/thermodynamic factors that coordinate biochemical events could interfere with the pathway that will be activated after DNA damage. Regarding the complexity of the DNA repair system, some authors consider the elucidation of these mechanisms of action a challenge (5).

Zebrafish (*Danio rerio*) is an organism commonly used as a biologic model. Besides characteristics like easy handling in laboratory, low maintenance costs, relatively rapid development, sequenced genome and a high number of larvae/embryos per hatch, this species has demonstrated similarities in many physiologic processes with mammals (13–15). This aspect allows zebrafish to be a suitable organism for use as a model for several human diseases (16). According to Lam *et al.* (15),

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zebrafish liver tumor possesses general molecular hallmarks of human cancer, showing deregulation in a large number of genes coding for proteins involved in cell cycle/proliferation, apoptosis, DNA replication and repair, and others. Many conserved components of different pathways from the DNA repair system have been described for zebrafish (12,17,18). Additionally, Sussman (19) reported that this species is highly competent in repairing UV radiation-induced damage.

Within this point of view, the aim of the present study was to evaluate the effects of UV-B exposure on the expression pattern of genes related to DNA repair system in hepatocytes of zebrafish (*D. rerio*), providing new information about the gene regulation mechanism responsible for UV response in this fish species.

MATERIALS AND METHODS

Cell line and culture conditions. The lineage of hepatocytes from zebrafish was obtained from the American Type Culture Collection (lineage ZFL; ATCC® number CRL-2643™). The cells were grown at 28°C in disposable plastic flasks containing RPMI 1640 (Gibco) medium supplemented with sodium bicarbonate (0.2 g L^{-1}), L-glutamine (0.3 g L^{-1}), Hepes (25 mM), β-mercaptoethanol (5×10^{-5} M), fetal bovine serum (10%; Gibco) and 1% of antibiotics and antimicotic (penicillin [100 U mL^{-1}], streptomycin [100 μg mL^{-1}], amphotericin B [0.25 μg mL^{-1}]; Gibco).

Treatment of cells (UV-B exposure). Cells (1×10^6 cells per well) were transferred to 24-well culture plates and allowed to attach for 48 h at 28°C. After this period, the medium was removed and the cells monolayer washed with PBS. The UV-B exposure was carried out in a volume of 1 mL of PBS per well. Cells were irradiated with different doses of UV-B (ranging from 0 to 70 mJ cm^{-2}) (VL-115 L, 30 W; Vilber Lourmat, France). The distance from the bulb source to the cell culture surface was 25 cm and the duration of the exposure varied from 5 to 60 s depending on the dose. This lamp produces 493 μW cm^{-2} UV-A and 1.195 mW cm^{-2} UV-B, with contamination of 0.113 μW cm^{-2} visible light and no contamination with UV-C (20). After irradiation, PBS was removed and 1 mL of fresh medium was added in each well. Cells were maintained at 28°C in the dark for 24 and 48 h. When the medium was removed, the cell monolayer was washed with saline phosphate buffer (Ca^{2+}- and Mg^{2+}-free; PBS) and then trypsin–EDTA (1.25 g L^{-1} trypsin and 0.2 g L^{-1} EDTA) was added. After trypsinization, the same volume of medium was added. The number of viable cells was measured by Trypan blue exclusion.

Analysis of gene expression. Only cells exposed to 0 and 23.3 mJ cm^{-2} of UV-B were used for expression analysis through the semiquantitative reverse transcription–PCR method (21). After 6, 12 and 24 h of irradiation, cells of three separate culture dishes were grouped (forming only one sample) and employed for total RNA isolation using TRIzol reagent (Invitrogen, Brazil) following the manufacturer's recommendations. For each experimental group, three to four samples were used for total RNA isolation. The total RNA extracted was quantified using the Qubit™ Quantitation Kit (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA according to the protocol of the 3′-RACE system (Rapid Amplification of cDNA Ends; Invitrogen) using the adapter primer provided with the kit. First-strand cDNA was then amplified by PCR using gene-specific primers (Table 1) designed from sequences available in the DDBJ/GenBankTM/EBI Data Bank using the Primer3 online program (22). PCR was carried out employing the *Platinum* Taq DNA Polymerase (Invitrogen) according to the following protocol: initial denaturation at 94°C for 2 min, followed by variable number of cycles (see Table 1) of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30–60 s (see Table 1) and a final extension period of 10 min at 72°C. To normalize the data, β-actin expression was used as an internal control. Tests using sequential dilutions of samples were done in order to assegurate that data are in the linear range of PCR (Fig. S1, Supplemental Material). The PCR products were analyzed after electrophoresis on 1% agarose gels employing the software *1DScan* (Scanalitica).

Table 1. Gene-specific primers used for RT-PCR analysis, NCBI accession numbers for sequences used in primer design and PCR-specific conditions used for each pair of primers (for more details, see Materials and Methods).

Gene	Mechanism	NCBI accession number	PCR-specific conditions		Forward primer (5′–3′)	Reverse primer (5′–3′)
			Extension time (s)	Cycles		
β-actin	Cytoskeleton	AF057040	30	25	CCCCTTGTTCACAATAACCT	TCTGTTGGCTTTGGGATTCA
Apex1	DNA repair (BER)	BC055545	30	31	GAGTTTCCTGATTTCTTCCTGGT	CATGTAGGTCCAGAAGGTGTAGG
Bax	Apoptosis	BC055592	40	30	GAGCTGCACTTCTCAACAACTTT	CTGGTTGAAATAGCCTTGATGAC
Bcl-2	Apoptosis	BC133848	40	31	TTGTGGAGAAATACCTCAAGCAT	GAGTCTCTCTGCTGACCGTACAT
CyclinG1	Cell cycle arrest	BC052125	40	30	TCTCTCCTTGACTCGATTCTTTG	AATATTCAACCAGGCACTTAGCA
DDB2	DNA repair (NER)	NM001083061	60	40	AAAAGACCGAATGAAGAAACTCC	TAGTAAGCAACTTGGTGCTGTCA
Gadd45α	Cell cycle arrest	NM001002216	30	35	TTGAAGAACCGTGTGGAGATAAC	TGTTCACTCGCAAGATATTGATG
Ku80	DNA repair (NHEJ)	AY877316	45	34	TGGAGGAGATTGAGAGAGAACTG	GTTTCATCATCATCGTTCAGACA
Nbs1	DNA repair (HR)	AY858826	60	31	GTCACCCTGTCTCAAGATATTCG	GAGGACATCACTGTTTCTTGGAC
Ogg1	DNA repair (BER)	XM001332667	30	37	ACAGAAAGGAAAAGTGGAAGAGG	TGAGAAAAGACACTCCACAGGAT
CDKI	Cell cycle arrest	CU062631	30	32	GATCCTACGTTCACTCGGTAATG	TTGGCTTGGTAGAAATCTGTGAT
p53	Cell cycle arrest	U60804	30	32	CTATAAGAAGTCCGAGCATGTGG	GGTTTTGGTCTCTTGGTCTTTCT
Rad51	DNA repair (HR)	BC062849	45	32	GATCCTGACAGAAGCTGCTAAAA	CACCTCTGCCTTTCCTAAGGTAT
XPC	DNA repair NER)	NM00104521	30	34	TATGGAGACCAAAAAGAACCTCA	CAGGTTTCGACTCAAGTGCTAGT

BER = base-excision repair; NER = nucleotide-excision repair; NHEJ = nonhomologous end-joining; HR = homologous recombination.

Statistical analysis. All experiments were performed at least in triplicates. Data were expressed as mean (±SE) and analyzed by ANOVA followed by the Tukey test. ANOVA assumptions (normality and homogeneity of variances) were previously checked. The significance level (α) was fixed at 0.05.

RESULTS AND DISCUSSION

Irradiation of zebrafish hepatocytes with different UV-B doses significantly ($P < 0.05$) reduced the number of viable cells in groups exposed to UV-B (from 17.5 to 70 mJ cm^{-2}) when compared with the control group (Fig. 1). Only the lowest dose (11.6 mJ cm^{-2}) was not able to significantly reduce viable cells number. When cells were analyzed under the microscope, hepatocytes exposed to 35 and 70 mJ cm^{-2} showed several cellular alterations (Fig. 2). The doses used here are lower than those used by Dong *et al.* (23) in zebrafish embryos (930 mJ cm^{-2}) but were similar to many doses found in the literature for diverse cell types (24–26). Based on these results, only the UV-B dose of 23.3 mJ cm^{-2} was used for gene expression experiments, because it was the highest used dose that affected viable cell number in 30% but produced no visual alteration on cell morphology.

It is known that the primary target of UV-B radiation is the DNA molecule, leading mostly to the formation of photoproducts like CPDs and 6-4PPs, which could activate the DNA repair system. Hence we analyzed the expression pattern of some genes involved in different mechanisms of this response, which are cell cycle arrest, damage repair and apoptosis (see Table 1). An important response observed after DNA damage is the activation of molecules involved in cell cycle arrest, which provides time for DNA repair. We also analyzed the expression of four genes (*p53*, *CDKI, Gadd45α* and *CyclinG1*) that could be acting in cell cycle arrest (Fig. 3). Among these, *p53* encodes a protein that has a key role in the response to DNA damage (8). In the present study, a significant ($P < 0.05$) induction in *p53* gene expression was observed only 24 h after the exposure to UV-B radiation (Fig.3). Nevertheless, some authors (27,28) have demonstrated a more rapid up-regulation of *p53* gene expression after exposure to other agents (like hydrogen peroxide and microcystin-LR) and in other organisms. However, this result is consistent with the fact that the primary response to DNA damage involves activation of the p53 protein predominantly through its phosphorylation by DNA damage-responsive kinases (29). Almost immediately after damage, an activation of the p53 protein present would be observed. Induction of *p53* gene transcription would only occur if the protein basal levels were not sufficient, for example after a prolonged period after damage occurrence. This idea is in agreement with the upregulation of *CDKI* (cyclin-dependent kinase inhibitor p21-like) gene expression 6 h after UV-B exposure observed in the present study (Fig.3), which is a primary mediator of p53-dependent G1 cell cycle arrest following DNA damage. Up-regulation of *p21* gene expression is a common response observed after DNA damage provoked not only by UV radiation but also by a wide range of agents, like ionizing radiation (30), hydrogen peroxide (27) and microcystin-LR (28). It is interesting to note that this gene showed a biphasic response, being up-regulated 6 h after UV-B exposure and down-regulated after 12 h, returning to control levels after 24 h (Fig. 3). This response could suggest some kind of negative feedback control.

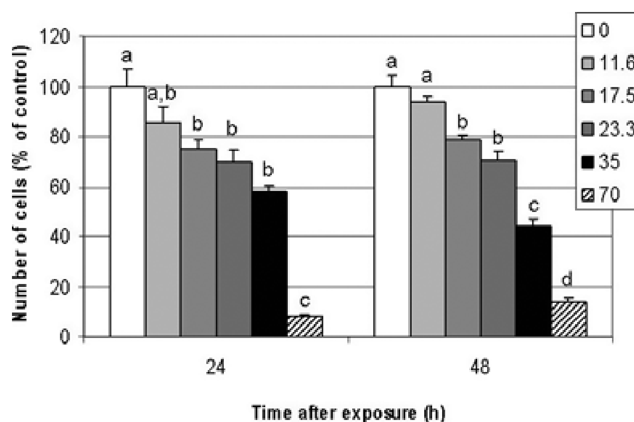

Figure 1. Number of viable cells 24 and 48 h after exposure to different UV-B doses (0, 11.6, 17.5, 23.3, 35 and 70 mJ cm^{-2}). Values are represented as percentage of control treatment. Data are expressed as mean ± SE (n = 3–8). Equal letters indicate absence of significant difference ($P > 0.05$) between samples in the same time after UV-B exposure.

Similar to *CDKI*, *cyclinG1* (also involved in cell cycle arrest) gene expression was also down-regulated 12 h after UV-B exposure (Fig. 3). However, 24 h after exposure this gene showed an up-regulation, similar to results found in the literature (27). On the other hand, *gadd45α* gene expression was altered (up-regulated) by UV-B only after 24 h (Fig. 3). According to Amundson *et al.* (29), *gadd45α* is a known p53 effector gene and is related to G2/M arrest and DNA repair. The late expression of *gadd45α* observed in the present study was also found by Zegura *et al.* (28) in HepG2 cells after microcystin-LR exposure. According to the authors, this late response could be related to its role in activation of the G2/M checkpoint following exposure to genotoxic stress or to the response to the type of DNA damage.

The second mechanism analyzed in the present study was the removal of cells that do not efficiently repair damage in the DNA molecule, with apoptosis being the most prominent route of cell inactivation after DNA damage. This is a defense mechanism in terms of nonrepaired DNA damage that often has harmful consequences such as chromosomal changes, gene mutations and malignant transformations (31). One mechanism that promotes apoptosis is related to members of the bcl-2 family, which is a large family of apoptosis regulators that either promote cell survival or facilitate cell death. In fact, according to several authors (32–34) heterodimerization between pro- and anti-apoptotic members of this family can neutralize one another's activity, suggesting that the relative concentration of one group *versus* the other greatly influences the response of cells after a death signal. Thus, expression of two members of this family was analyzed in the present study, one anti-apoptotic (*bcl-2*) and another pro-apoptotic (*bax*). In this context, *bcl-2* gene expression was down-regulated at all times analyzed and the expression of the pro-apoptotic gene *bax* was not significantly altered ($P > 0.05$) after UV-B exposure (Fig. 4a). In zebrafish, dietary exposure to methylmercury, a substance that can cause DNA damage through oxidative stress generation, increased expression of *bax* gene in the liver only after

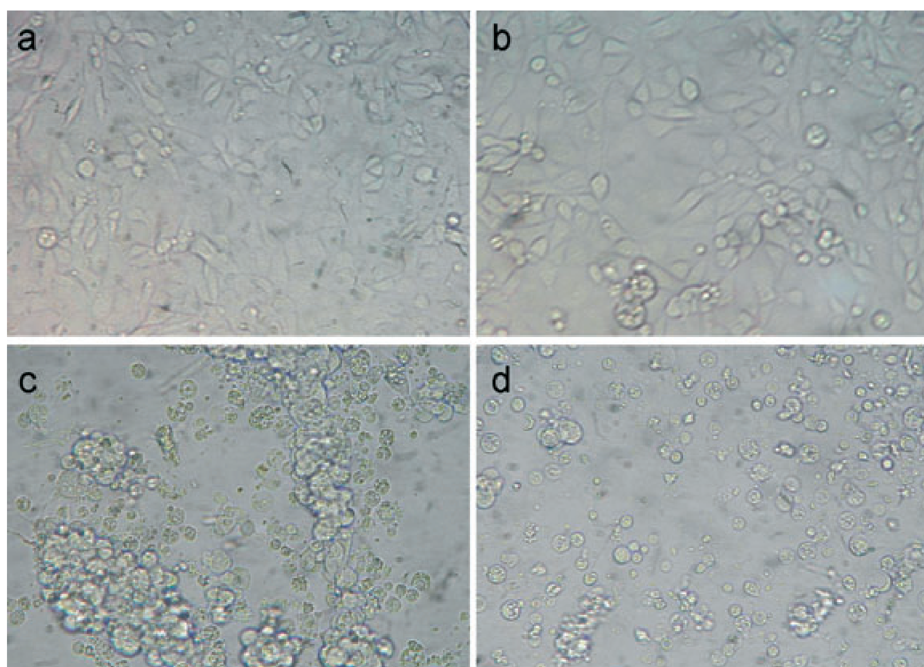

Figure 2. Effects of UV-B exposure on cell morphology. Cells were exposed to 0, 23.3, 35 and 70 mJ cm^{-2} of UV-B (a, b, c and d, respectively) and analyzed under microscope after 24 h of exposure.

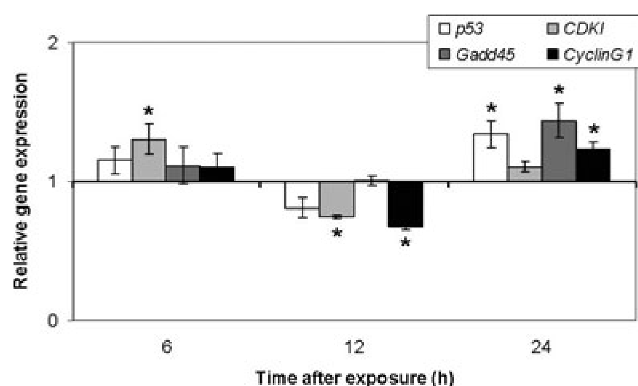

Figure 3. Kinetics of expression of some genes involved in cell cycle arrest (*p53, CDKI, gadd45* and *cyclinG1*) after exposure to 23.3 mJ cm^{-2} UV-B. Values are relative to control group and expressed as mean ± SE (n = 4). Significant difference (P < 0.05) between control and cells exposed to UV-B at the same time is indicated by an asterisk.

63 days (35). The ratio between expression of *bax* and *bcl-2* was increased after UV-B exposure (Fig. 4b), which could be causing an increase in apoptosis level after DNA damage caused by UV-B exposure. Others studies observed the same pattern of increase in *bax/bcl-2* ratio after DNA damage (28,30). Various members of the bcl-2 family are known as p53 target genes, including *bax*. However, the expression pattern of this gene was not coincident with *CDKI*, another p53 target gene. These results, apparently contradictory, could be explained by the fact that low levels of activated p53 are enough to promote *p21* gene expression, and only when p53 becomes accumulated above a particular threshold can it activate the transcription of pro-apoptotic genes (31). According to Latonen and Laiho (1), this mechanism could be related to a lower affinity of p53 to binding sites on apoptosis-related genes compared to targets related to cell cycle arrest.

The results of gene expression observed here could explain the effects of reduction in cell number after UV-B exposure observed in Fig. 2. In this sense, this effect of UV-B exposure could be produced both by a cell cycle arrest, through action of p21, gadd45 and cyclinG1, and/or by apoptosis, through members of the bcl-2 family.

Concerning DNA repair, the third mechanism involved in DNA repair system analyzed in the present study, several molecules are involved in this process. According to the type of lesion generated in the DNA molecule, a specific route of DNA repair will be activated. In this sense, some genes involved in the main routes of DNA repair (nucleotide-excision repair, NER; base-excision repair, BER; nonhomologous end-joining, NHEJ; and homologous recombination, HR) were selected for analyses (see Table 1).

Among the several genes involved in NER, the proteins XPC and p48 (product of *DDB2* gene) are involved in the recognition of UV-induced lesions in DNA, being XPC more related to recognition of 6-4PPs and p48 to CPDs (36). According to Ford (8), expression of both these genes is regulated by p53 and the kinetics of this induction occurs between 6 and 24 h after the damage has occurred. Moreover, this increase in gene expression could be important for replenishing the level of these proteins degraded during the process of DNA repair (8). In the present study, expression of *XPC* and *DDB2* genes was up-regulated after UV-B exposure (Fig. 5), with maximum values being found 6 h after exposure. *DDB2* showed the highest up-regulation (after 6 h—fold induction of 6.53 ± 0.60 when compared with the control) among all genes analyzed here. However, 24 h after the exposure it was not possible to measure the expression of this

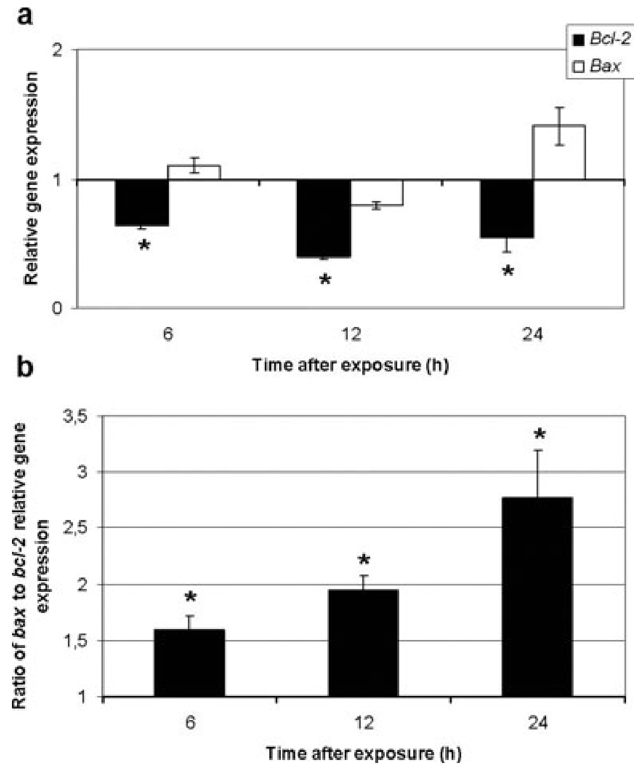

Figure 4. Effects of UV-B exposure on (a) expression of *bcl-2* and *bax* genes; and (b) ratio of *bax* to *bcl-2* relative gene expression. Values are relative to control group and expressed as mean ± SE ($n = 4$). Significant difference ($P < 0.05$) between control and cells exposed to UV-B at the same time is indicated by an asterisk.

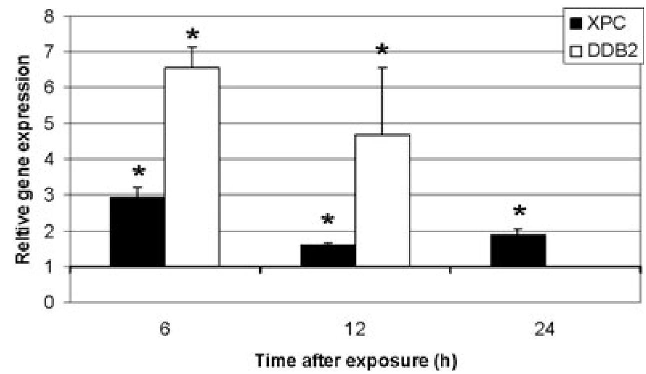

Figure 5. Effects of UV-B exposure on expression of *XPC* and *DDB2* genes. Values are relative to control group and expressed as mean ± SE ($n = 4$). Significant difference ($P < 0.05$) between control and cells exposed to UV-B at the same time is indicated by an asterisk.

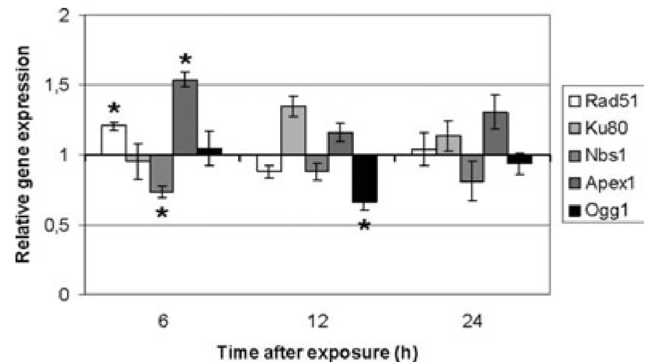

Figure 6. Effects of UV-B exposure on expression of DNA repair genes (*Rad51, Ku80, Nbs1, Apex 1* and *Ogg1*). Values are relative to control group and expressed as mean ± SE ($n = 4$). Significant difference ($P < 0.05$) between control and cells exposed to UV-B at the same time is indicated by an asterisk.

gene, because its level was extremely low, including in the samples exposed to UV-B. These results are in agreement with the literature cited before and could be an important tool for monitoring UV-B exposure.

Besides CPDs and 6-4PPs, other lesions in the DNA molecule could be produced, which in turn would activate different mechanisms of repair. Concerning this, five other genes involved in other routes of DNA repair were analyzed in the present study (BER: *Apex1* and Ogg1; HR: *Rad51* and *Nbs1*; NHEJ: *Ku80*) (Fig. 6). Expression of *Nbs1* and *Ogg1* was down-regulated 6 and 12 h after UV-B exposure, respectively. On the other hand, expression of *Rad51* and *Apex1* was up-regulated only 6 h after the exposure. *Ku80* gene expression was unaltered by UV-B exposure. Some of these results are in agreement with the literature. For example, up-regulation of *Rad51* gene expression was found in the liver of zebrafish after exposure to methylmercury (35). However, the same response was not observed when fishes were exposed to cadmium (10). The results of the present study could indicate that other types of DNA lesions were produced after hepatocyte exposure to UV-B radiation. This idea is supported by the up-regulation of the *Apex1* gene. The protein coded for this gene is involved in repair of oxidized bases through the pathway known as base excision repair (37). According to Ellinger-Ziegelbauer *et al.* (38), *Apex1* gene is inducible only after exposure to nongenotoxic carcinogens, which could act through oxidative stress generation. As mentioned before, UV-B is a genotoxic carcinogen as it can act mainly through direct interaction with DNA. However, it has been described that UV-B is able to induce the formation of 8-oxo-7,8-dihydroguanine, the most frequent base lesion produced after oxidative damage to DNA (2). Therefore, the up-regulation of genes like *Apex1* after UV-B exposure found here could indicate the production of oxidative lesions in DNA molecule.

The results of the present study demonstrate some aspects of the DNA repair system in zebrafish hepatocytes after UV-B exposure. Perhaps the main mechanism observed in this work is the central role of p53 in response to UV-B-induced DNA damage in this species. First, UV-B exposure could have produced an activation of p53 protein present in cells, making it possible to act as a transcription factor altering the expression of some genes involved in DNA repair system. In this sense, this probable rapid p53 activation could have increased the transcription of *p21*, *XPC* and *DDB2* genes and decreased the transcription of the *bcl-2* gene. Thereafter, levels of the p53 protein were augmented through induction of *p53* gene transcription (24 h after exposure), which would be related to the observed increase in expression of *gadd45α* and *cyclinG1* genes. The patterns of *bcl-2*, *p21*, *gadd45* and *cyclinG1* gene expression could explain the reduction in the number of viable cells observed after UV-B exposure. In this sense, UV-B exposure could lead to a viable cell number decrease by cell

cycle arrest or by cell death through apoptosis. Besides alterations of genes involved in cell cycle arrest (*p53*, *CDKI*, *gadd45α*, *cyclinG1*), apoptosis (*bcl-2*) and repair of the main types of UV-B-induced lesions in DNA (*XPC*, *DDB2*), zebrafish hepatocytes exposed to UV-B radiation also showed variations in the expression of genes related to the repair of other types of DNA lesions. In this context, UV-B exposure led to an up-regulation of the *Apex-1* gene after 6 h of exposure, which could be related to generation of oxidative lesions in the DNA molecule. All these results provide additional evidence about the mechanisms of DNA repair system in hepatocytes of zebrafish after exposure to UV-B radiation and it could be a useful tool in terms of comparison with the mechanisms observed in other species, mainly within mammals.

Acknowledgements— Juliana Z. Sandrini is a postgraduate student financed by the Brazilian CNPq. This study was supported by MCT/CNPq.

SUPPLEMENTAL MATERIALS

The following supplemental materials are available for this article:

Figure S1. Effects of sequential dilution of three different samples in B-actin gene expression.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/full/10.1111/j.1751-1097.2008.00422.x

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