Zebrafish as a ''Biosensor''? Effects of Ionizing Radiation and Amifostine on Embryonic Viability and Development

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Abstract

The zebrafish (*Danio rerio*) has emerged as a popular vertebrate model system for cancer and treatment-related research. Benefits include ease of care, rapid development, optical clarity of embryos, which allows visualization of major organ systems, and opportunities for genetic manipulation. However, specific parameters of radiation sensitivity have not been systematically documented. We investigated the effects of radiation and a radiomodifier on zebrafish viability and embryonic development. Embryos were exposed to γ-radiation (5, 10, or 20 Gy) at sequential times postfertilization and serially assessed for viability and morphologic abnormalities. As expected, lethality and morphologic perturbations were more pronounced earlier in embryogenesis and with higher radiation doses and were partially reversed by amifostine. The effects of radiation and concurrent treatment with amifostine on the developmental organization of the eye and brain were striking. Radiation resulted in hypocellularity and disorganization of the cellular layers of the retina, effects partially reversed by amifostine, as well as lens opacification. Radiation strikingly reduced the volume of brain, but the volume loss was substantially blocked by amifostine. Increased terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling signal was noted in both the irradiated eye and brain, but reduced by amifostine. Finally, irradiating embryos resulted in caspase activation detectable in 96-well microplates, which was proportional to the number of embryos and radiation dose; the degree of activation was markedly reduced by amifostine. These results together suggest the power and versatility of the zebrafish in assessing the effects of radiation and radiomodifiers on organ and tissue development. (Cancer Res 2006; 66(16): 8172-81)

Introduction

The zebrafish is a diploid organism that possesses many advantages for studying human diseases, as well as the effects of therapeutic and other environmental agents. The human and zebrafish genomes share considerable homology, including conservation of most DNA repair–related genes. Large numbers of fish can be maintained in a small space at relatively low cost, and paired matings typically produce hundreds of embryos. Embryonic development is rapid, with major organ systems such as the eyes, brain, heart, liver, muscles, bone, and gastrointestinal tract evident within 48 hours postfertilization (hpf). Because embryogenesis occurs outside the mother and in water, the effects of drug and radiation exposure are easy to assess and do not require parental sacrifice. Genetic manipulations are relatively simple in the zebrafish because genetic knockouts and transgenic animals can be created by the microinjection of morpholino-modified oligonucleotides complementary to the target gene or mRNA expressed from the target gene, respectively (1). These factors together contribute to the growing popularity of zebrafish for cancer-related studies.

Zebrafish embryos are ideal for evaluating genotoxic stress as well as radiation-related studies. The mutagenic effects of radiation on zebrafish embryos were noted in early reports that found greater radiosensitivity before the midblastula transition (2), but these studies did not assess the effects of different radiation doses or categorize the types and severity of mutations. More recently, MacAleer et al. (3) found increased susceptibility of early embryos to radiation and showed the radioprotective effects of amifostine, a free radical–scavenging thiol that is presently in clinical use. Bladen et al. (4) cloned the zebrafish orthologue of human Ku80, involved in DNA strand-break rejoining, and confirmed its role in the DNA damage response.

We have systematically investigated the developmental time and dose dependency of zebrafish embryo viability with the effects of radiation. In addition to the susceptibility of early embryos to the lethal and morphology-altering effects of radiation, we found striking effects of radiation on the cellular organization of the eye and development of the brain, organs that are often dose-limiting for clinical therapeutic radiation. These effects were correlated with increased unrepaired DNA strand breaks and were partially reversed by amifostine. Because the small size of zebrafish embryos renders them suitable for microplate-based assays, we investigated the effects of radiation on caspase activation of irradiated embryos in 96-well plates. Radiation resulted in a dose-dependent increase in caspase activation. Interestingly, caspase activation induced by radiation was blocked by amifostine. These observations together support the usefulness of zebrafish for investigating radiation-related effects and radiomodifying agents on embryonic viability and development.

Materials and Methods

Zebrafish maintenance and embryo handling. Zebrafish use and handling was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Wild-type adult zebrafish were obtained from Carolina Biological Supply Co. (Burlington, NC) and local pet stores (PetSmart, Philadelphia, PA) and were maintained according to standard operating procedures (5). Zebrafish were kept at 28.5°C on a 14-hour day/10-hour night cycle. Adult fish were kept segregated by sex and mated in embryo collection tanks (Aquatic Habitats, Apopka, FL). Embryos from these breedings were harvested at the one-cell to two-cell stage during

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development (6) and were washed and sorted at 25.0°C before incubation at 28.0°C for the duration of the experiments. Embryos were sorted using a dissecting light microscope (Nikon SMZ1000, Florham Park, NJ) and were kept at a concentration of 10 to 40 embryos per well in standard six-well polystyrene tissue culture plates (Costar, Corning, NY) with 5 mL of standard zebrafish E3 embryo medium (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L $CaCl_2$, 0.33 mmol/L $MgSO_4$, 10^{-5}% methylene blue; ref. 7) buffered with 2 mmol/L HEPES (Sigma, St. Louis, MO) in each well, which was changed at 24-hour intervals. To ensure uniform removal of the chorion, unhatched embryos at 48 hpf were incubated with 30 μg/mL Pronase (Sigma) in E3 for ~30 minutes at 25.0°C before gentle agitation with a disposable plastic transfer pipette. All embryos were subsequently washed thoroughly in fresh E3 before being returned to tissue culture plates. Nonviable embryos were promptly removed over the observation period to prevent fungal and bacterial growth.

Embryo irradiation and amifostine exposure. Protocols involving the irradiation of zebrafish embryos were approved by the Department of Environmental Health and Radiation Safety at the University of Pennsylvania. Embryos at 2, 4, 6, 8, or 24 hpf were mock irradiated or exposed to single fractions of 640 kVp γ-irradiation at room temperature using a J.L. Shepherd Mark I ^{137}Cs irradiator in standard six-well polystyrene tissue culture plates with 5 mL of E3 at a distance of 20 cm. The dose rate (1.33 Gy/min) was determined by Fricke dosimetry. Treated embryos were exposed to 4 mmol/L amifostine [MedImmune Oncology (Gaithersburg, MD) and Sigma], dissolved in E3, for 30 minutes before radiation exposure.

Experimental analyses. Survival of each embryo was continually assessed from the point of fertilization up to 144 hpf or the conclusion of each experiment. All observations were made with light microscopy. For the first 24 hpf, survival was defined through the assessment of appropriate cell division using the method described by Kimmel et al. (6). After 24 hpf, cardiac contractility defined continued survival. Survival was calculated as a percentage of viable embryos to total number of embryos for each treatment group and survival curves represent the mean of three separate experiments. Radioprotection ratios were calculated as a survival ratio with amifostine-pretreated embryos as the numerator and non-amifostine-pretreated embryos as the dominator. Viability assays used a total of 4,439 individual embryos.

Morphologic analysis occurred at 24-hour intervals and representative embryos were photographed at 72 hpf. Dechorionated embryos were anesthetized with a 1:100 dilution of 4 mg/mL tricaine (3-aminobenzoic acid ethyl ester methanesulfonate, 1% Na_2HPO_4, pH 7; Sigma) and placed onto a glass slide containing 3% methylcellulose for photography. Images were acquired at ×20 magnification with either a Nikon Coolpix 8500 (Nikon SMZ1000) or Photometrics Coolsnap digital camera (Leica MZ125) using iVision software for the Macintosh. For histologic evaluations, embryos were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue slices (5 μm) were stained with H&E, assessed with a BX41 microscope at ×40 magnification, and photographed using a SPOT camera with SPOT advanced software. At least 20 embryos from each treatment group were assessed. The diameter of the eye was defined as the distance from the pigmented epithelium of one pole to the opposite pole, and assessed at day 3.

For terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays, embryos were fixed in 4% paraformaldehyde for 24 hours, followed by copious washing with 1% PBS. The embryos were subsequently embedded in paraffin and sectioned onto glass slides. The slides were dewaxed, rehydrated, and incubated with Proteinase K (19.7 mg/mL; Roche Diagnostics Corporation, Indianapolis, IN) in 10 mmol/L Tris-hydrogen chloride (pH 7.5) for 30 minutes at room temperature. The slides were then washed and blocked with 0.25 mg/mL bovine albumin in 1% PBS for two 10-minute incubations. After the bovine albumin was drained, the slides were incubated in the dark for 60 minutes at 37°C with TUNEL reaction mixture (Roche). Fluorescence imaging was subsequently done with a ×100 PlanNeofluor objective mounted on a Nikon TE-200 microscope equipped with epifluorescence optics. Images were captured with a Hammamatsu CCD camera controlled with IP LabSpectrum v2.0.1 software (Scanalytics, Inc., Rockville, MD).

For assays involving detection of bioluminescent imaging of caspase activity in zebrafish embryos, the embryos were maintained at densities ≤20 embryos/5 mL medium and exposed to radiation at 4 hpf. Immediately before imaging at 6 hpf, the treated embryos were plated onto fresh black 96-well plates with clear bottoms (Corning) at a concentration of 10 embryos per well (except where noted) with the appropriate Caspase-Glo reagents (Promega Corporation, Madison, WI) added. Caspase-Glo 8 and Caspase-Glo 9 lyophilized substrates (LETD-aminoluciferin or LEHD-aminoluciferin, respectively) were prepared in accordance with the instructions of the manufacturer (Promega) in the appropriate lysis buffer. To assess the effects of radiomodifiers, embryos were exposed to radiation and assessed with the Caspase-Glo 9 assay in the presence of one of the following: embryo medium only (control); 4 mmol/L amifostine; pancaspase inhibitor benzyloxycarbonyl-valine-alanine-aspartate fluoromethylketone (z-VAD-fmk; Bachem Bioscience, King of Prussia, PA) at 50 μmol/L as a negative control; or 0.1 μmol/L staurosporine (Sigma) as a positive control. All were dissolved in E3 for 30 minutes before radiation exposure.

For all experiments, ~2 hours after incubation with Caspase-Glo preparations (or individual components), the 96-well microplates containing the embryos were imaged for luminescence with the Kodak 4000MM Imaging System (Eastman-Kodak, Rochester, NY). Images were captured and rendered in negative so that wells emitting more light are therefore shown as darker wells than those not emitting light. The photon emission was analyzed with Kodak Molecular Imaging Software, version 4.0, for the Macintosh. Arbitrary luminescent units were defined by software, with individual wells manually defined as the region of interest with background values subtracted.

Statistical analyses were done with VassarStats[3] (courtesy of Richard Lowry) and Microsoft Excel (Office XP Professional). Comparison of the averages of two groups was done with Student's t test whereas that of three groups was done with ANOVA analysis. Unless otherwise indicated, error bars indicate SD. Statistical significance was defined at $P < 0.05$.

Results

The age of the embryos at the time of irradiation and radiation dose determine subsequent survival and morphology, but both can be modified by amifostine. We investigated the effects of ionizing radiation on zebrafish embryonic survival after irradiation at different periods following fertilization, using >4,000 zebrafish embryos divided between the different treatment groups. Irradiating embryos at 2 hpf with 5 Gy had a profound effect on survival, with <25% of irradiated embryos still alive at 72 hpf with very few surviving for longer periods (Fig. 1*A*). When amifostine was present at the time of irradiation, survival of the embryos was substantially improved at all subsequent time points (e.g., ~35% of embryos were alive at 72 hours). Interestingly, in addition to improving overall survival, amifostine also seemed to ameliorate some of the effects of radiation on morphology. The few embryos that survived 5 Gy of radiation were markedly malformed, with shortened and curved bodies, and exhibited malformations of the head and eye along with notable pericardial edema. These effects were also seen in embryos that survived irradiation in the presence of amifostine, but these morphologic aberrations appeared less severe (representative embryos shown to the right of the survival curves in Fig. 1*A*). Finally, amifostine alone in the absence of irradiation had no appreciable effect on either survival or morphology.

The resistance of the embryos to radiation increased substantially with embryonic age. Approximately 25% of embryos treated

[3] http://faculty.vassar.edu/lowry/VassarStats.html.

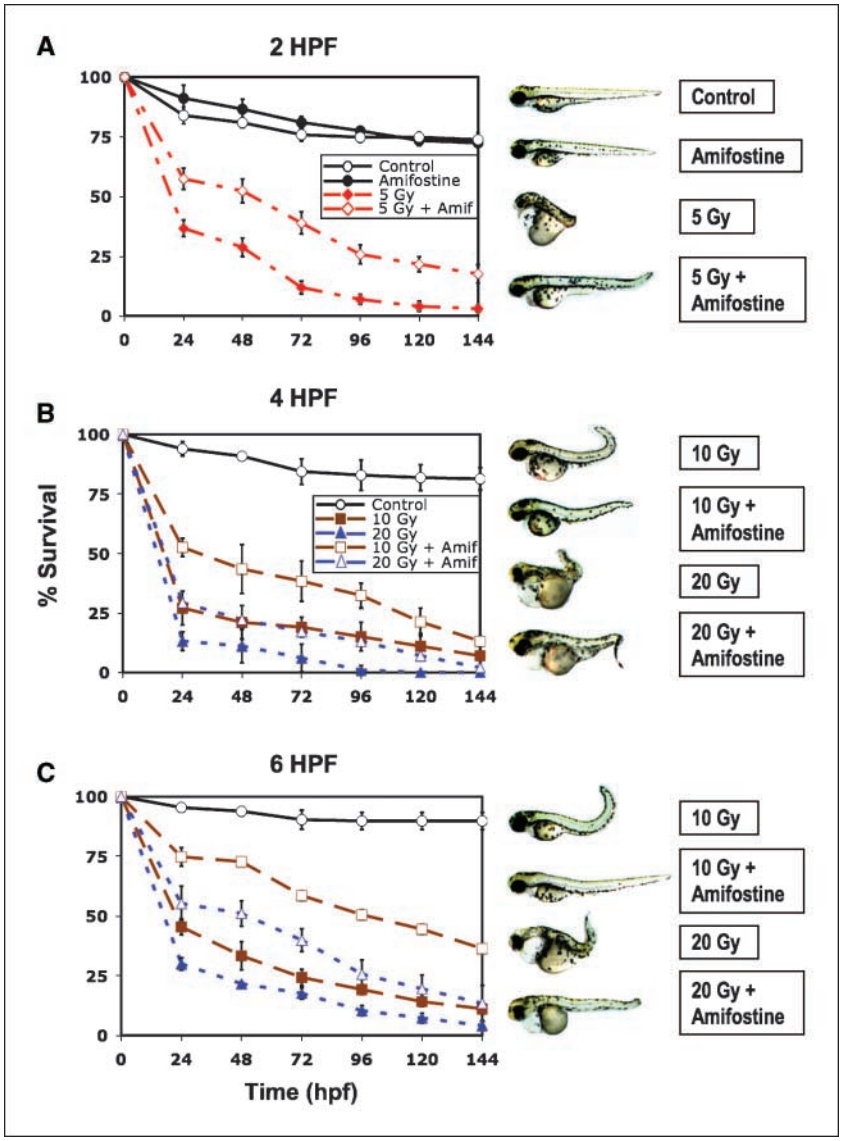

Figure 1. The effects of ionizing radiation on zebrafish survival in the presence and absence of amifostine. *A,* zebrafish embryos at 2 hpf were mock irradiated (*Control*, ○), mock irradiated plus amifostine (*Amifostine*, ●), and irradiated with 5 Gy (♦) or 5 Gy plus amifostine (◇). The percentage of viable zebrafish embryos was determined for each treatment group at 24-hour intervals up to 144 hpf. Morphology was also evaluated for each group at 72 hpf, with representative photographs (taken at ×20 magnification) shown to the right of the survival curves. *B,* as described in (*A*) with the exception that radiation was delivered at 4 hpf at the following doses: 10 Gy (■), 10 Gy plus amifostine (□), 20 Gy (▲), or 20 Gy plus amifostine (△). *C,* as described in (*B*) with the exception that radiation was delivered at 6 hpf in doses identical to (*B*). Legend used is the same as in (*B*). *Points,* the mean percentage derived from three experiments; *bars,* SE. In the experiment shown in this figure, 1,678 embryos were used, with the number of embryos in each treatment group as follows: 2 hpf—*Control*, 199 embryos; *Amifostine*, 99; *5 Gy*, 100; *5 Gy + Amifostine*, 98. 4 hpf—*Control*, 197; *10 Gy*, 100; *20 Gy*, 99; *10 Gy + Amifostine*, 99; *20 Gy + Amifostine*, 98. 6 hpf—*Control*, 196; *10 Gy*, 99; *20 Gy*, 97; *10 Gy + Amifostine*, 99; *20 Gy + Amifostine*, 98.

with 10-Gy radiation at 4 hpf remained alive at 72 hpf (Fig. 1*B*). Therefore, despite a dose of radiation that was twice as high, the proportion of surviving embryos irradiated at 4 hpf was higher than that irradiated at 2 hpf. Although the proportion of embryos that survived 10-Gy irradiation at 4 hpf was higher than those receiving 5 Gy at 2 hpf and at all subsequent time points, severe morphologic abnormalities were still noted in the surviving embryos. However, the survival and morphologic appearance of embryos irradiated with 10 Gy were both improved by amifostine. To further extend these studies, we also assessed the susceptibility of the embryos to 20-Gy irradiation. No embryos irradiated with 20 Gy at 4 hpf survived longer than 120 hpf and most died with severe morphologic abnormalities. With amifostine, a small number of embryos survived 20 Gy of irradiation but with obvious phenotypic abnormalities consistent with radiation injury.

The trend towards increased survival was also noted when the embryos were irradiated even further from the time of fertilization. Approximately 35% of embryos treated with 10-Gy radiation at 6 hpf remained alive at 72 hpf; survival increased to ~60% when the irradiation occurred in the presence of amifostine (Fig. 1*C*). The proportion of embryos that survived 20-Gy irradiation was also higher for age-matched amifostine-treated embryos than those irradiated without amifostine. Once again, whereas amifostine increased the proportion that survived this dose of radiation, it did not prevent the resultant morphologic abnormalities.

Following these discoveries, we sought to better describe the conditions under which amifostine improved the survival of irradiated embryos because the respective proportions of surviving embryos may change over time and depend on the radiation dose. We therefore defined the radioprotection ratio as the proportion of embryos that survived a given dose of ionizing radiation in the presence of amifostine, divided by the proportion that survived the same radiation dose without amifostine. We repeated the experiments described in Fig. 1, but with all three radiation doses administered to embryos that were at 2, 4, 6, 8, or 24 hpf. The embryos were irradiated in the absence or presence of amifostine, and the survival of each group of embryos was assessed every 24 hours until 144 hpf.

These experiments showed that the radioprotection ratio of amifostine increased in embryos irradiated at 2 hpf with 5 Gy and was greatest (ratio, 5.9) at the conclusion of the experiment

(144 hpf; top portion of Table 1). To a lesser extent, the ratio also increased with the passage of time in embryos irradiated with 5 Gy at 4 and 6 hpf. In contrast, little appreciable radioprotection was noted in embryos irradiated with 5 Gy at 8 or 24 hpf. These results indicate that the radioprotective effects of amifostine seem to be greatest in the youngest embryos at this dose of radiation. However, at this dose of radiation, little radioprotection could be noted in embryos irradiated beyond 8 or 24 hpf.

Embryos at 2 hpf were very susceptible to the lethal effects of 10- or 20-Gy radiation, with little to no survival after 24 hours. Whereas amifostine increased survival after these higher doses at a number of time points, the absolute numbers of surviving embryos were very low. Consequently, radioprotection ratios >20 were likely not "clinically meaningful" and simply reflected the lack of survival of embryos irradiated without amifostine (middle portion of Table 1). At 4 or 6 hpf, a higher proportion of embryos survived, which was further increased when the irradiation occurred in the presence of amifostine. Amifostine resulted in radioprotection ratios of 2 to 3 in embryos irradiated with 10 Gy at 4 to 6 hpf, with a smaller degree of radioprotection noted in embryos irradiated with this dose at 8 hpf and little radioprotection with irradiation at 24 hours hpf. These results suggest that the protective effects of amifostine are most pronounced during early embryogenesis and diminished in older embryos.

The effects of amifostine in embryos irradiated with 20 Gy were also complex. In embryos irradiated at 2 or 4 hpf, radioprotection ratios were meaninglessly high at 120 hours, as all the embryos irradiated at this dose of radiation without amifostine had succumbed (lower portion of Table 1). In embryos irradiated at 6 and 8 hpf, radioprotection ratios of 3.3 and 3.0, respectively, were noted by 144 hpf and the ratios increased with the passage of time. Interestingly, even at this dose of radiation, radioprotection from amifostine was diminished in embryos irradiated at 24 hpf.

These experiments together suggest that the radioprotective effects of amifostine on zebrafish embryo survival depend on the age of the embryo at the time of irradiation and the dose delivered. At a lower dose of 5 Gy, the greatest radioprotection is noted in the youngest embryos, and the radioprotection is diminished in embryos irradiated at a later stage. At 10 and 20 Gy, amifostine seemed to confer the highest degree of radioprotection to embryos irradiated up to 8 hpf. At all doses of radiation, comparatively less radioprotection was seen in embryos irradiated at 24 hpf.

Effects of ionizing radiation on brain and eye development. In the experiments described in Fig. 1, irradiation of zebrafish embryos resulted in a number of specific morphologic abnormalities consistent with those reported by MacAleer et al. (3) and Bladen et al. (4). These included curvature of the spine, shortening of the overall length of the body, pericardial edema, inhibition of yolk sac resorption, micro-ophthalmia, and microcephaly (Fig. 2). Although it may be tempting to find correlates of these observations with late effects of radiation noted in mammals (such as kyphosis/lordosis, growth retardation, pericardial edema/pericarditis, etc.), we elected to focus on the effects of radiation on the eye and brain. Radiation-related effects such as cataract formation, retinal degeneration/atrophy, blindness, and microcephaly are feared complications that have been reported after inadvertent or therapeutic exposure to radiation (8–14).

We found that radiation led to marked reduction in the volume of the tectum of the brain, such that a large portion of the skull case was devoid of brain matter. This was evident at 10 Gy and was even more marked after 20 Gy (Fig. 3*A*). Interestingly, the telencephalon and cerebellum seemed considerably less affected by radiation. It was more difficult to judge the effect on the diencephalon because it is contiguous with and lacks obvious anatomic boundaries that separate it from the tectum. Finally, we found that the effects of radiation were moderated by amifostine.

Table 1. Radioprotection ratio

	0 hpf	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf	144 hpf	
2 hpf	1.0	1.6	1.8	**3.3**	**3.7**	**5.5**	**5.9**	5 Gy
4 hpf	1.0	1.5	1.6	1.7	1.9	1.9	1.9	
6 hpf	1.0	1.1	1.3	1.3	1.4	1.4	1.4	
8 hpf	1.0	1.1	1.2	1.2	1.1	1.1	1.1	
24 hpf	1.0	1.0	1.1	1.0	1.0	1.0	1.0	
2 hpf	1.0	**8.4**	**>20**	**>20**	**>20**	**>20**	**>20**	10 Gy
4 hpf	1.0	1.9	**2.1**	**2.0**	**2.1**	1.9	1.9	
6 hpf	1.0	1.6	**2.2**	**2.4**	**2.6**	**3.1**	**3.3**	
8 hpf	1.0	1.3	1.2	1.2	1.2	1.5	1.6	
24 hpf	1.0	1.0	1.1	1.0	1.1	1.1	1.1	
2 hpf	1.0	**17.5**	**>20**	**>20**	**>20**	**>20**	**>20**	20 Gy
4 hpf	1.0	**2.2**	**2.0**	**2.8**	**13.0**	**>20**	**>20**	
6 hpf	1.0	1.8	**2.4**	**2.3**	**2.5**	**2.7**	**3.3**	
8 hpf	1.0	1.2	1.3	1.5	**2.1**	**2.6**	**3.0**	
24 hpf	1.0	1.0	1.1	1.1	1.1	1.2	1.2	

NOTE: Amifostine radioprotection ratios for irradiated zebrafish embryos exposed at various hpf to 5, 10, or 20 Gy. Survival was evaluated in 24-hour increments up to 144 hpf. The radioprotection ratio represents survival at the specific time point of the group exposed to amifostine divided by the survival of the group exposed to radiation only [radioprotection ratio = survival with amifostine / survival without amifostine]. Values >2.0 are shown in bold. Values higher than 20 are listed as >20, usually representing instances where no embryos survived radiation only (and for which the radioprotection ratio therefore has a denominator of zero).

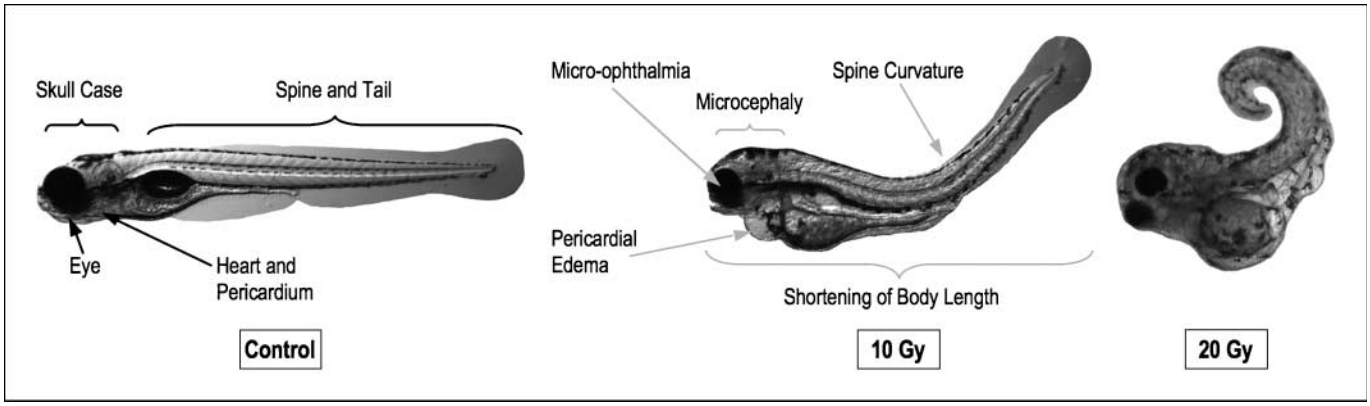

Figure 2. Morphologic effects of ionizing radiation in the developing zebrafish. Zebrafish embryos were exposed at 4 hpf to 0, 10, or 20 Gy radiation. After irradiation, the embryos were incubated at 28 °C and photographed at 144 hpf. The skull case, eye, heart and pericardium, and spine and tail are labeled on the control embryo (*black arrows* and *brackets*). Specific morphologic aberrations of these areas are labeled for the embryo irradiated with 10 Gy (*gray arrows* and *bracket*). Micrographs were taken of representative embryos at ×40 magnification.

Unlike the embryos treated with radiation alone, the skull case of embryos irradiated with amifostine was not devoid of brain tissue in the area of the tectum and the other regions of the brain also seemed to be less perturbed.

The effect of radiation on the eye was also marked. The diameter of the eyes of control unirradiated embryos at 72 hpf was 185 ± 21 μm, compared with 141 ± 26 μm in embryos irradiated with 10 Gy and 170 ± 19 μm in embryos treated with 10 Gy in the presence of amifostine ($P < 0.001$). The cellular organization of unirradiated control eye shows concentric layers representing the zebrafish retina (rods and cones), which is immediately interior to the pigmented epithelium followed sequentially by concentric rings representing the outer plexiform layer, the bipolar cell layer, the inner plexiform layer, the ganglion cell layer, and finally the lens (representative eyes of each treatment group at 144 hpf are shown in Fig. 3*B*). In contrast to these distinct cellular layers detectable in the control eye, the organization of the eye after irradiation was considerably disrupted. Although thinner than in the control, the pigmented epithelium could be still be discerned in the irradiated eye (Fig. 3*B, 10 Gy, white arrow*). However, it was difficult to distinguish most of the remaining cellular layers. The rod and cone layer was not concentrically organized and the inner plexiform layer, which was conspicuous in the control eye, was nearly absent after radiation (*arrowheads, 10 Gy*). Likewise, the prominent round nuclei of the ganglion cell layer could not be discerned. Finally, radiation resulted in lens opacification (*black triangle, 10 Gy*).

The presence of amifostine at the time of irradiation partially preserved the cellular organization. The layer of rods and cones could be identified immediately internal to the pigmented epithelium (Fig. 3*B, 10 Gy + Amifostine, white arrow*). Both the outer and inner plexiform layers could be readily identified (*10 Gy + Amifostine, solid arrow*), albeit thinner than in the unirradiated control eye. Whereas the ganglion cell layer was retained after irradiation and amifostine, there seemed to be fewer cells than in the control eye and the lens remained opacified (*10 Gy + Amifostine, black triangle*). These results suggest that the effects of irradiation on the zebrafish eye include disruption of the cellular organization, with depletion of specific layers as well as lens opacification. Amifostine partially prevents these changes but does not prevent cellular depletion and thinning of specific structures, nor does it seem to protect against lens opacification.

Ionizing radiation leads to increased cell death in the eye and brain of the zebrafish embryo. The profound effects of radiation on the development of the eye and brain may be due to increased cell death, decreased growth, or both phenomena. To begin to distinguish between these possibilities, we did TUNEL analyses. This highly specific assay detects double-strand DNA breaks, which may be inflicted by DNA-damaging agents such as radiation or may arise during the course of programmed cell death (i.e., apoptosis; refs. 15, 16). Although the assay does not detect all forms of double-strand DNA breaks (such as blunt-ended breaks), TUNEL labeling is often regarded as a standard marker of cell death. Indeed, with TUNEL analysis, Bladen et al. (4) noted apoptosis in the zebrafish hindbrain and the peripheral nervous system after irradiation. We assessed whether signal could be detected elsewhere in the brain, particularly in the tectum. As expected, unirradiated control brain showed no TUNEL labeling (Fig. 4*A, top*). In contrast, 10-Gy radiation led to substantial TUNEL labeling throughout the tectum (average of 57 ± 12 TUNEL-positive cells per field; Fig. 4, *middle*); however, the areas of TUNEL signal were significantly reduced by amifostine [although isolated TUNEL-positive cells could still be detected (average of 7 ± 3 TUNEL-positive cells per optical field; $P < 0.001$; Fig. 4, *bottom, arrowheads*)].

Radiation resulted in impressive TUNEL labeling within the eye (Fig. 4*B, middle, arrowhead*), as well as in the tissues outside of the eye (*arrow*). TUNEL labeling within the eye, as well as in the tissues outside, was found in each of 20 embryos that were individually assessed (including data shown). Amifostine resulted in markedly diminished TUNEL labeling of tissues outside of the eye (Fig. 4*B, bottom, arrow*). Unexpectedly, labeling of cells within the eye could still be detected (*arrowhead*) although the intensity of the staining for most of the cells seemed to be less than in eyes irradiated in the absence of amifostine. The physiologic basis of the persistent low TUNEL positivity within the eye despite amifostine remains to be fully elucidated, but may represent sublethal damage or signal in a subset of the cells. Nonetheless, in all embryos irradiated in the presence of amifostine, TUNEL labeling of cells outside the eye was greatly diminished.

Ionizing radiation leads to increased caspase activation detectable in a microplate-based assay of zebrafish embryos. TUNEL reactivity is often noted in settings involving caspase activation, such as during apoptosis (15, 17). The strong TUNEL signal that was induced by radiation suggested that it might be

fruitful to assay for caspase activation. Caspase activity may be induced by radiation and might therefore serve either as a direct or indirect marker for assessing the effects of radiation. We chose an assay that could be completed rapidly and which required minimal sample manipulation, therefore limiting interoperator variability, and which was based on cleavage of proluminescent probes by activated caspase (Caspase-Glo; Promega). Caspases have in common the specificity for cleaving characteristic tetrapeptide sequences after an aspartic acid residue (e.g., D-x-x-D, where D represents aspartic acid; ref. 18). In the assay, when endogenous activated caspases are present, probes consisting of caspase-recognition peptides fused to luciferin are cleaved after the aspartic acids, thereby releasing luciferin. When the luciferin is exposed to luciferase (also part of the assay), light is emitted in proportion to the degree of caspase activation.

We first confirmed the specificity of the assays for caspase-8 and caspase-9, as well as whether or not they would show activity under appropriate conditions. The assay was done in 96-well plates, individual wells of which can comfortably fit many zebrafish embryos. With the same number of embryos (10) in each well, we assessed the effect of mock irradiation and irradiation with 10 Gy, with all components of the assay; only the buffer component of the assay; the powder component; or all components of the assay in wells devoid of embryos, the latter of which served as an additional control. Radiation resulted in light emission with both the caspase-8 and caspase-9 assays (although substantially more light was associated with the caspase-9 under otherwise identical assay conditions; top row of wells in Fig. 5*A*). In contrast, light was not detected after irradiation of embryos in wells with only individual components of the assays (either

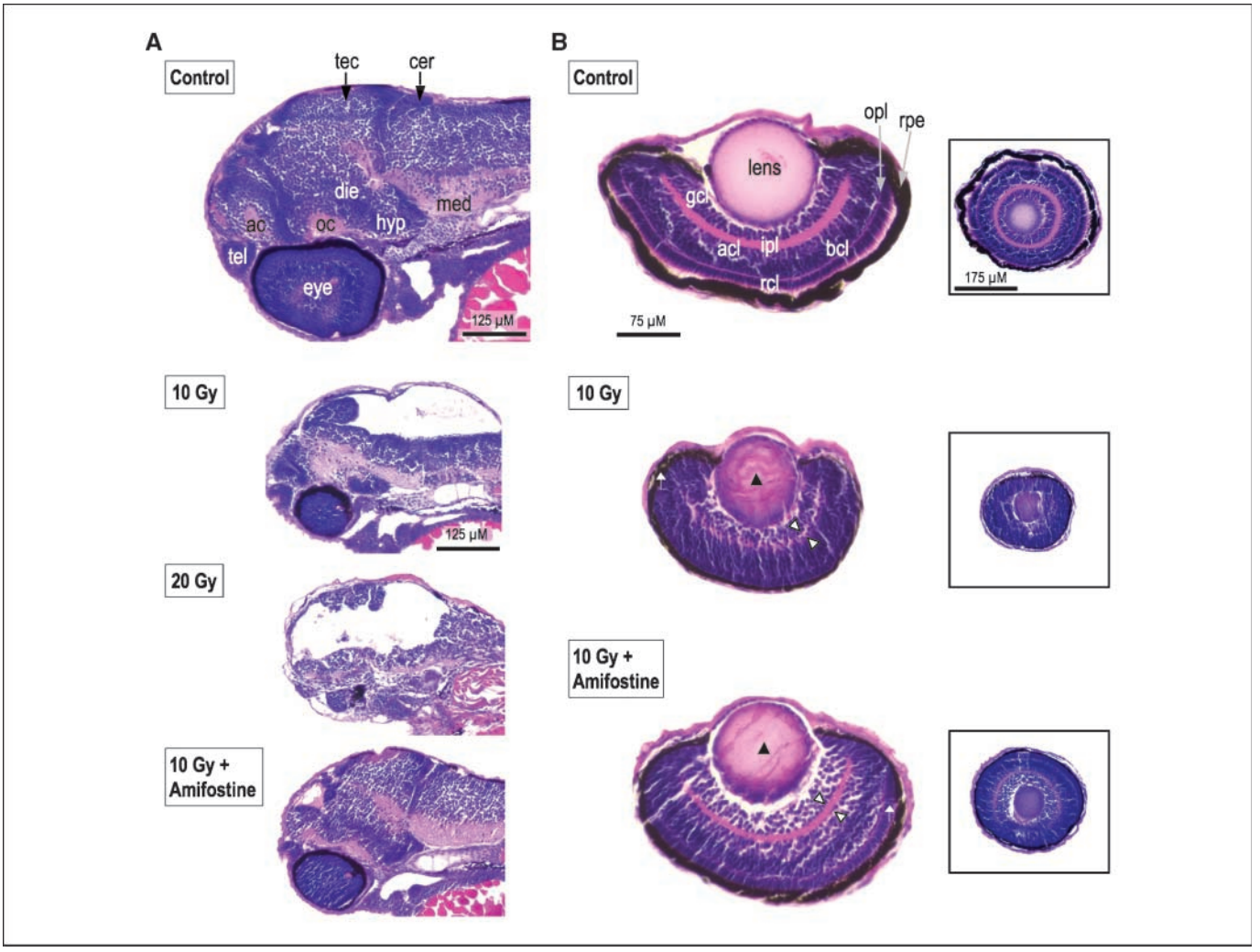

Figure 3. Effects of ionizing radiation on the morphology of the developing central nervous system and eye in zebrafish embryos. *A,* H&E-stained sagittal sections of the cranial structures of representative surviving zebrafish embryos at 144 hpf after exposure at 4 hpf to 0 (*Control*), 10, 20, or 10 Gy with amifostine. Layers are indicated as follows: *ac,* anterior commissure; *cer,* cerebellum; *die,* diencephalon; *hyp,* hypophysis; *med,* medulla; *oc,* optic chiasm; *tec,* tectum; *tel,* telencephalon. The embryo treated with 10 Gy shows overall loss of brain volume, particularly of the tectum and diencephalon. The embryo treated with 20 Gy shows even greater loss of brain volume, as well as severe effects on the eye, whereas the embryo treated with 10 Gy in the presence of amifostine shows reduced loss of brain volume. *B,* H&E-stained transverse sections through the central retina of surviving zebrafish embryos at 144 hpf after exposure at 4 hpf to 0 (*Control*), 10, or 10 Gy with amifostine. *Insets,* representative eyes from identically treated embryos from a coronal perspective. In the image of the control eye (*Control*), layers are indicated as follows: *acl,* amacrine cell layer; *bcl,* bipolar cell layer; *gcl,* ganglion cell layer; *ipl* and *opl,* inner and outer plexiform layer, respectively; *rcl,* rods and cones layer; *rpe,* retinal pigmented epithelium. The eye of an embryo treated with 10 Gy (*10 Gy*) shows overall loss of volume as well as loss of the defined architecture; beyond the lens (*black triangle*), other structures such as the inner plexiform layer and outer plexiform layer (*white arrowheads*) are difficult to distinguish. The eye of the embryo treated with 10 Gy in the presence of amifostine (*10 Gy + Amifostine*) shows reduced loss of volume, as well as preservation of recognizable structures such as ganglion cells, inner and outer plexiform layers (*arrowheads*), bipolar cells, rods and cones, and pigmented epithelium (*white arrow*).

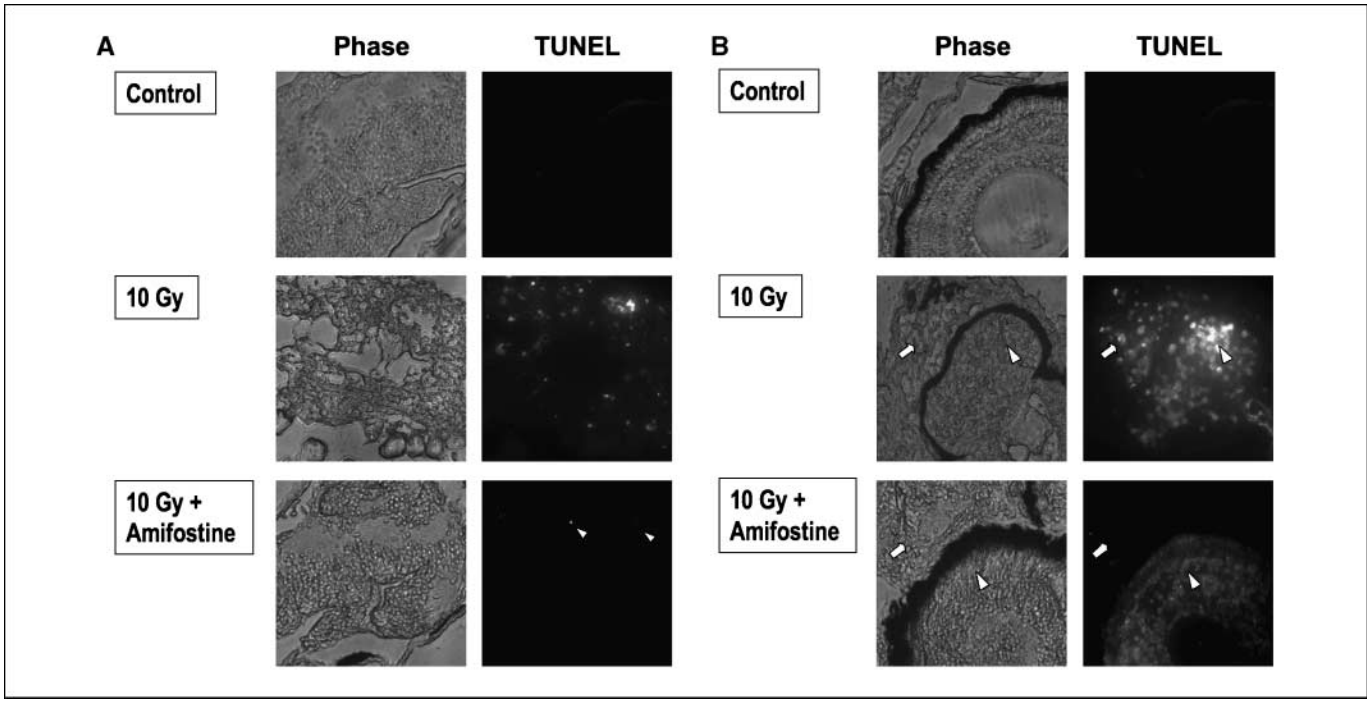

Figure 4. Irradiation results in widespread TUNEL labeling of brain tissue and cells both inside and outside the developing eye. Zebrafish embryos at 4 hpf were mock irradiated (*Control*) or exposed to 10 Gy, or 10 Gy in the presence of amifostine. At 144 hpf, surviving zebrafish from each treatment group were mounted, sectioned, and assayed for TUNEL labeling. Representative phase-contrast (*Phase*) images and the same fields imaged under fluorescence (*TUNEL*) from each treatment group are shown for both the developing central nervous system (*A*) and eye (*B*). *A,* control unirradiated zebrafish show no TUNEL signal (*top*) in this region of brain tissue whereas those irradiated with 10 Gy (*middle*) show diffuse, punctuate TUNEL labeling. The brain tissues of zebrafish embryos irradiated with 10 Gy in the presence of amifostine (*bottom*) show substantially reduced TUNEL signal, but individual cells that are TUNEL positive can be seen (*arrowheads*). *B,* control unirradiated zebrafish show no TUNEL signal (*top*) in the region of developing eye shown, whereas those irradiated with 10 Gy (*middle*) show substantial TUNEL labeling of cells inside the eye (*arrowhead*), as well as in cells outside the eye (*arrow*). Zebrafish irradiated with 10 Gy in the presence of amifostine show reduced TUNEL signal, especially in the cells outside the eye (*bottom; arrow*). However, many cells inside the eye still seem to show TUNEL signal (*arrowhead*). All images were taken at ×1,000 magnification.

buffer or powder alone; second and third rows of wells in Fig. 5*A*). Finally, no light was detected after irradiation when all assay components but no embryos were present (bottom row of wells).

These initial results were encouraging, but we wished to determine whether caspase activity resulting from the irradiation of fewer embryos could be detected. The assay was therefore repeated, but with 2, 5, 10, or 20 embryos in each well, which were treated with 10 Gy as in the previous experiment. We found that the amount of light emitted by the assay, particularly that for caspase-9, correlated with the number of embryos in the assay (Fig. 5*B* and first four sets of columns in the histogram shown in Fig. 5*C*). The assay was of sufficient sensitivity to detect the effect of radiation on just two embryos. Increased light emission with the caspase-8 assay was also noted, but the levels were lower than with the corresponding wells for caspase-9. We also assessed the levels of light emission with the higher radiation dose of 20 Gy. At this dose, the amount of light emission also increased with increasing number of embryos (last three sets of columns in the histogram in Fig. 5*C*). Interestingly, although the amount of light emitted was highest for the 20 embryos that were irradiated with 20 Gy, the amount of light emitted was clearly not twice the amount of light emitted by the same number of embryos irradiated with 10 Gy. This suggests that there may be an "optimal" radiation dose range, beyond which the amount of caspase activated by the radiation may not be proportional to the radiation dose.

To further extend these results, we investigated the effects of different doses of radiation in the presence and absence of amifostine. As additional controls, we included the effects of a synthetic pancaspase inhibitor (z-VAD-fmk) and staurosporine (a phosphatase inhibitor that is often used to induce apoptosis in a wide variety of cell lines; refs. 19, 20). Radiation resulted in dose-dependent increases in light emission resulting from caspase-9 activation (top row of wells shown in Fig. 5*D* and first set of columns in the histogram shown in Fig. 5*E*). As expected, z-VAD-fmk largely abolished the caspase activation induced by radiation (third row of wells shown in Fig. 5*D* and third set of columns in Fig. 5*E*). We were surprised to find that amifostine also substantially reduced the caspase activation induced by radiation (second row of wells in Fig. 5*D* and second set of columns in Fig. 5*E*). Finally, as expected, staurosporine induced substantial caspase activation (bottom-most well in the microplate and last column of the histogram) in the absence of radiation.

These results suggest that caspase activation in zebrafish embryos induced by radiation may be readily detectable by measuring the light emitted from a convenient reagent-based assay that results in light emission that is proportional to the number of embryos and the radiation dose. The small size of the embryos allows the assays to be completed in 96-well microplates. Finally, convenient positive (staurosporine) and negative controls (z-VAD-fmk) are available, which confer additional confidence in the results of the assay.

Discussion

We have systematically characterized the effects of ionizing radiation on the survival and development of zebrafish embryos, including changes in the eye and brain, and determined that these effects are associated with increased cellular death. We have shown that both overall survival and perturbations in development are ameliorated by amifostine. These results are consistent with the observations of McAleer et al. (3) and Bladen et al. (4). We have further shown that the irradiation of zebrafish embryos results in caspase activation in a dose- and embryo-dependent fashion, which can be efficiently measured in a microplate-based assay. These findings collectively attest to the versatility and usefulness of zebrafish as a powerful model system for investigating radiation and other anticancer treatments.

We have recapitulated in the zebrafish embryos a number of feared complications of radiation that have been described in mammals, including humans. Consideration of the risk of inducing retinal and optic atrophy, lens opacification, and microcephaly often (and appropriately) limits the delivery of therapeutic radiation in the clinic. Our findings that some of these complications might be at least partially ameliorated by radiomodifiers, such as amifostine, merit further investigation. Other changes induced by radiation, including effects on body morphology and organ systems such as the heart, have not been formally quantified in our investigations and may also represent useful end points to assess. Such studies would add to the well-established value of zebrafish-based studies for genetic and cellular analyses (3, 4, 21, 22).

Our preliminary observation that amifostine may confer radioprotection to an extent that may depend on the cellular or tissue-type merits confirmation, especially in other animal models. It may be possible that the ability of different cell types to tolerate

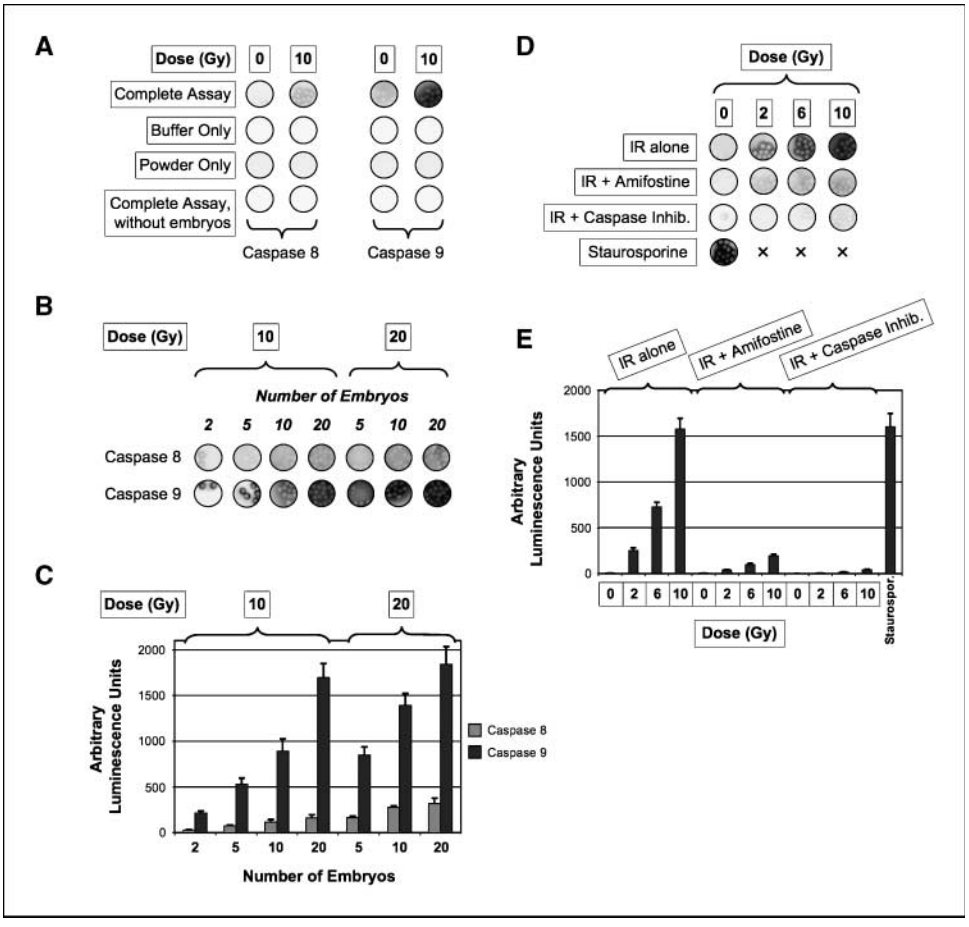

Figure 5. Bioluminescent imaging of caspase activation in zebrafish embryos. *A,* zebrafish embryos (10 per well) were mock irradiated (0 Gy) or irradiated with 10 Gy at 4 hpf. Ten embryos from the respective treatment groups were immediately placed into individual wells of a 96-well microplate, followed by a 30-minute exposure, under gentle agitation, to either the combined components of the LETD-aminoluciferin (caspase-8) or LEHD-aminoluciferin (caspase-9) assay reagents (*Complete Assay*); lysis buffer only (*Buffer Only*); reagent reconstituted without lysis buffer (*Powder Only*); or the combined components of the respective caspase-8 and caspase-9 assay reagents in the absence of embryos (*Complete Assay, without embryos*). The LETD- or LEHD-aminoluciferin powder was reconstituted in E3 embryo medium. Imaging was done 1 hour after mock irradiation or irradiation. For maximal clarity and contrast, the embryo-containing wells are shown as negative images (i.e., emission of light results in darker images). *B,* zebrafish embryos were irradiated with 10 or 20 Gy, and the indicated numbers of embryos from either treatment group were placed into individual wells of a 96-well microplate. All embryos were then assayed with the complete components of the caspase-8 and caspase-9 assay reagents at 1 hour after irradiation. *C,* histograms showing the relative levels of emitted signal from the experiment shown in (*B*), displayed in arbitrary units of luminescence. *D,* zebrafish embryos (10 per well) were mock irradiated (0 Gy) or irradiated with 2, 6, or 10 Gy at 4 hpf in medium only (*IR alone*), in the presence of amifostine (*IR + Amifostine*), in the presence of a pancaspase inhibitor (*IR + Caspase Inhib.*), or mock irradiated but exposed to staurosporine (*Staurosporine*). Ten embryos from each respective treatment group were immediately placed into individual wells of a 96-well microplate, followed by a 30-minute exposure under gentle agitation to the combined components of the caspase-9 assay reagents. Imaging was done 1 hour after mock irradiation or irradiation. *E,* histograms showing the relative levels of emitted signal from the experiment shown in (*D*), displayed in arbitrary units of luminescence. All wells are shown as negative images.

radiation may be affected by amifostine or other radiomodifiers due to differences in the repair capacity, in the intracellular pathways that are affected, or in the ability to absorb the radiomodifying agent. On a tissue or organ level, there may be differences in perfusion, pH status, or hypoxia, any or all of which may influence the efficacy of amifostine (23–26). There may also be a threshold dose of radiation, beyond which amifostine is largely ineffective. Amifostine may confer organ-specific levels of radioprotection, causing the agent to be more effective in preventing the radiation-induced death of specific tissues or organs (23, 24, 27–29). We cannot exclude the possibility that the mechanisms by which amifostine leads to increased survival are different among embryos at different ages.

It is tempting to speculate that the susceptibility of newly fertilized embryos to radiation may be due to the lethal effects of perturbed or abrogated organ development. Gastrulation, the first definable point at which organ development begins, is generally described as occurring at about 5 to 7 hpf in the zebrafish embryo (6). The susceptibility of the embryos to the lethal effects of radiation seems to diminish after this point, which would support the hypothesis that events occurring at or before gastrulation are particularly radiosensitive (2). However, the causes of death may be multifactorial. It should also be noted that the assays described here were conducted over a brief period of time, and the survival "curves" were concluded at just 6 days postfertilization. It is possible that lower doses of radiation would ultimately lead to decreased survival with increased "follow-up." Radiation may lead to alterations of behavior or responsiveness to the environment that might impede the ability to feed, which may not immediately result in death but rather in an ultimately lethal failure to thrive (8, 10, 13, 14). Effects on the gastrointestinal system are likely underestimated here, as perturbations in the absorptive capacity or gastrointestinal motility may require sensitive functional assays or periods of observation longer than that described in this work.

The survival curves that describe viability after radiation over time are quantitative, but because they require 5 days or more to complete, the assay may not be suitable for rapid analyses. Morphologic changes can be quantitated, but these are end points that require additional processing of the treated embryos and a considerable degree of technical skill. These limitations also apply to TUNEL analyses. The proluminescent caspase activation assays may therefore be the most intriguing candidate to rapidly assess for the lethal effects of radiation because it can be completed within hours of radiation exposure and requires relatively few manipulations beyond the addition of reagents. The small size of the zebrafish embryos and reagent-based nature of the caspase activation assays (which do not require removal of the embryos during the experiment) may be done in 96-well microplates. These factors also suggest that this assay might be optimized for high-throughput screening, with automated equipment to add reagents and to shake, incubate, and image zebrafish embryo-containing microplates.

Whereas it was gratifying that we could show cytoprotective effects of amifostine in zebrafish embryos, the clinical use of amifostine in humans has been limited in some clinical settings by the requirement for parenteral administration, as well as by side effects that are likely mediated by hypersensitivity reactions, including hypotension, pruritus, and flushing (30). Consequently, the identification or development of novel agents of comparable or greater cytoprotective qualities with fewer side effects—perhaps assisted through zebrafish-based studies—would be greatly welcomed. The parameters we have established should facilitate future investigations into potentially novel radiomodifiers and strategies to increase as well as moderate the effects of treatment.

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