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A detailed characterization of congenital defects and mortality following moderate X-ray doses during neurulation

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Background: Both epidemiological and animal studies have previously indicated a link between in utero radiation exposure and birth defects such as microphthalmos, anophthalmos, and exencephaly. However, detailed knowledge on embryonic radiosensitivity during different stages of neurulation is limited, especially in terms of neural tube defect and eye defect development.

Methods: To assess the most radiosensitive stage during neurulation, pregnant C57BL6/J mice were X-irradiated (0.5 Gy or 1.0 Gy) at embryonic days (E)7, E7.5, E8, E8.5, or E9. Next, the fetuses were scored macroscopically for various defects and prenatal resorptions/deaths were counted. In addition, cranial skeletal development was ascertained using the alcian-alizarin method. Furthermore, postnatal/young adult survival was followed until 5 weeks (W5) of age, after X-irradiation at E7.5 (0.1 Gy, 0.5 Gy, or 1.0 Gy). In addition, body and brain weights were registered at adult age (W10) following X-ray exposure at E7.5 (0.1 Gy, 0.5 Gy).

Results: Several malformations, including microphthalmos and exencephaly, were most evident after irradiation at E7.5, with significance starting respectively at 0.5 Gy and 1.0 Gy. Prenatal mortality and weight were significantly affected in all irradiated groups. Long-term follow-up of E7.5 irradiated animals revealed a reduction in survival at 5 weeks of age after high dose exposure (1.0 Gy), while lower doses (0.5 Gy, 0.1 Gy) did not affect brain and body weight at postnatal week 10.

Conclusions: With this study, we gained more insight in radiosensitivity throughout neurulation, and offered a better defined model to further study radiation-induced malformations and the underlying mechanisms.

KEYWORDS
eye defect, neural tube defect, radiation, X-ray, mortality

1 | INTRODUCTION

Neural tube defects (NTDs) and eye defects (EDs) are believed to primarily arise from abnormal neural tube closure and disturbed early eye development, which can be caused by certain physical factors (Jablonski, 1999; Moretti, Bar-Oz, Fried, & Koren, 2005; Wertelecki et al., 2016) and teratogens such as valproic acid (Ehashi et al., 2014; Tanoshima et al., 2015) or various industrial and agricultural compounds (Brender & Weyer, 2016).

In terms of physical factors, several studies already identified the risks of exposure to ionizing radiation during pregnancy and stipulated a causal link with several congenital malformations (reviewed in [De Santis et al., 2005, 2007;

Jacquet, 2004; Verreet, Rangarajan et al., 2016; Verreet, Verslegers et al., 2016]). For example, in prenatally irradiated survivors of the Chernobyl disaster, the prevalence of both NTDs (e.g., exencephaly; exposed brain tissue) and EDs (e.g., microphthalmos; reduced eye size) was found to be increased (Burrow et al., 1964; Wertelecki, 2010; Wertelecki et al., 2014, 2016). NTDs (26.1/10 000) and microphthalmos (2.5/10 000) have a higher prevalence in the Polissia region (in the Rivne province) as compared to the rest of the Rivne province (respectively 16.4/10 000 and 0.8/10 000 between 2000 and 2009) that was less contaminated with radioisotopes after the Chernobyl disaster (European Surveillance of Congenital Anomalies [EUROCAT] Final Activity Report 2002–2003; Wertelecki, 2010; Wertelecki et al., 2014). The EUROCAT registers (2004–2008) support the increased prevalence of NTDs in Ukraine (Khoshnood, Greenlees, Loane, & Dolk, 2011). In Turkey, NTD occurrence also increased from an average of 21.2/10,000 to 43.9/10,000 the first 2 years following the disaster, which may be due to the radioactive contaminants originating from the Chernobyl disaster (Mocan, Bozkaya, Mocan, & Furtun, 1990). However, a big setback to the aforementioned epidemiological studies is the lack of dose estimates of pregnant women living in Cs-137 contaminated regions. In terms of international radiation protection standards, whole body count investigations noted that 48% of pregnant women in northern Polissia incorporated Cs-137 above the tolerated limit (2008–2011). In contrast, non-Polissia residents only exceeded the limit by 0.1%. Indeed, daily radiation activity uptake in the Polissia region (e.g., locally grown vegetables) was 268.25 Bq, which is above the upper permissible limit of 210.00 Bq (Wertelecki et al., 2014). There is, however, controversy about these findings since the EUROCAT registers did not demonstrate a detrimental impact of the Chernobyl disaster on birth-defect prevalences in western Europe (Dolk & Nichols, 1999). In contrast, a recent study suggested that fallout from the Fukushima disaster may have resulted in an increased prevalence of several NTDs at the west coast of the USA (Mangano & Sherman, 2015). In addition to this, a limited number of other studies also discussed radiation-induced congenital defects in an epidemiological setting, but it is crucial to note that the methodology of some studies was not ideal and neither was the size of the cohort. In addition, a more thorough screening in some affected regions after the Chernobyl and Fukushima disasters cannot be excluded and would generate false data (reviewed in [Akar, 2015]). A possible dose-dependency for defects such as NTDs is still under considerable debate (Dolk & Nichols, 1999). More studies are required to further support a causal link between prenatal exposure to ionizing radiation following the Chernobyl and Fukushima disasters, and the induction of NTDs and EDs.

The link between A-bomb survivors and NTDs and EDs is even less clear (Neel et al., 1956). In Hiroshima, only the incidences of microcephaly and mental retardation have been correlated to distance of the mother from the explosion epicenter (all cases were within 1200 m of the epicenter), if exposure occurred during the first 20 weeks of pregnancy (Plummer, 1952). Comparable observations were made in the Nagasaki A-bomb survivors (Yamazaki, Wright, & Wright, 1954). For a weighted uterine dose of 1.0 Gy and higher, the risk of severe mental disability was higher than 40%, in particular for those irradiated between weeks 8 and 15 after ovulation (Otake & Schull, 1998). Even though no conclusive increase in macroscopic EDs was observed following the atomic bombings, a decreased visual acuity was, however, found in in utero irradiated Japanese A-bomb survivors, hinting at more subtle persistent eye defects, following exposure to 0.5 Gy or higher during the first trimester (Burrow et al., 1964).

In terms of dose and dose rate, the A-bomb survivors differed strongly from the Chernobyl victims. The dose rates for a-bomb survivors were considerably higher than those of people living in radioisotope-contaminated regions in Ukraine, many years after the disaster. Fetal dosimetry, which extends upon the DS02 system, estimated the absorbed dose of the 8-week old brain between 0.356 and 0.851 Gy when the mother was at 1500 m from the hypocenter at Hiroshima or Nagasaki (Chen, 2012). These estimates are supported by the observation that the dose was often high enough to induce acute radiation syndrome in mothers with malformed children (Burrow et al., 1964). There are currently no accurate fetal dose estimations for pregnant women living in regions contaminated by radioisotopes from the Chernobyl and Fukushima disasters. The discrepancy between the outcomes of the Chernobyl disaster and the atomic bombings in terms of NTD and ED induction remains unclear. However, the current evidence nonetheless merits further research into radiation-induced congenital defects such as NTDs and EDs.

Birth defects such as microphthalmos and exencephaly result from complex deregulations of cellular mechanisms, where etiology is often complex and multifactorial (reviewed, respectively, in [Verma & Fitzpatrick, 2007] and [Copp, Stanier, & Greene, 2013]). Anomalous cell death (e.g., apoptosis or autophagy) (Cecconi, Piacentini, & Fimia, 2008; French et al., 2013; Marcos et al., 2015; Nakamura, Pichel, Williams-Simons, & Westphal, 1995; Zhang et al., 2010), proliferation and differentiation (Greene, Massa, & Copp, 2009; Iida et al., 2011; Jaramillo-Rangel et al., 2013; Ozeki & Shirai, 1998; Ozeki, Ogura, Hirabayashi, & Shimada, 2001; Zhang et al., 2013) are some of the mechanisms previously proposed to induce such defects, and interestingly, are also known to be affected by ionizing radiation (reviewed in [Bernhard, Maity, Muschel, & McKenna, 1995; Szumiel, 1994]). Yet no evidence for a direct link between radiation-induced NTDs/EDs and said mechanisms has been reported so far.

To support the aforementioned epidemiological data, various animal studies have been performed to better understand

the effect of prenatal radiation exposure on short- and long-term central nervous system (CNS) defects. Already more than 60 years ago, Russel studied the effects of acute high-dose radiation exposure throughout gestation with relatively long 24 hr time intervals, and suggested that the neurulation period was highly sensitive to X-irradiation (≥2 Gy), for example, EDs. The goal of these studies was to characterize radiation sensitivity throughout the entire gestational period, with a long time interval in-between acute irradiations (24 hr) (Russell 1950, 1956). However, since neurulation is a highly dynamic process, characterized by rapid differentiation, migration and formation of functional structures (as is reviewed in [Bazin-Lopez, Valdivia, Wilson, & Gestri, 2015; Nikolopoulou et al., 2017]), it is crucial to better characterize radiation sensitivity during the different phases of neurulation. To this end we decided that it was necessary to expand on the aforementioned studies and use shorter and better defined time intervals for radiation sensitivity characterization, whilst also using radiation doses at levels more relevant to potential exposures of pregnant women in situations of medicine (e.g., CT) and accidents. Furthermore, due to the focus of most recent studies on radiation-induced defects at later developmental stages, that is, during neurogenesis (E11–E17) (Quintens et al., 2015; Verreet et al., 2015; Verreet, Rangarajan et al., 2016; Verreet, Verslegers et al., 2016), there is a general lack of information on the impact of irradiation during neurulation, which starts at E7.5.

As such, our study aimed at unraveling the effect of irradiation at the time of neurulation on neural tube and eye development. Pregnant mice were irradiated with 0.1, 0.5, or 1.0 Gy at short intervals during the critical stages of neurulation, to pinpoint the most radiosensitive period during neurulation to develop EDs, NTDs and to affect prenatal/postnatal mortality/viability. Our findings thereby complement the current lack of knowledge on dose-dependent and stage-specific radiation-induced congenital anomalies, and will be crucial for future mechanistic studies.

2 | MATERIALS AND METHODS

2.1 | Macroscopic study and evaluation of prenatal mortality

All animal experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the local ethical SCK•CEN/VITO (ref. 02–012), University of Antwerp and KU Leuven committees. C57Bl/6 J were purchased from Janvier (Bio Services, Uden, The Netherlands) and housed under standard laboratory conditions (12-hr light/dark cycle). Food and water were available ad libitum. Female mice were coupled during a 2-hr time period in the morning, at the start of the light phase (7.30 hr until 9.30 hr), in order to ensure synchronous timing of embryonic development. Females showing a mating plug were randomly assigned to the different groups (control [0.0 Gy], 0.1 Gy, 0.5 Gy, or 1.0 Gy of X-rays). Acute single-dose X-irradiation was performed at specific gestational stages (embryonic day (E) 7, E7.5, E8, E8.5, or E9). A maximum of seven unanesthetized mice were placed within a disk-shaped Plexiglas box (20 cm diameter, 5 cm height) at the irradiation facility shortly before irradiation, with each individual animal housed in a small subcompartment, thus limiting free movement. The irradiation was performed using a Pantak HF420 RX operating at 250 kV, 15 mA, 1mm Cu filtration at a dose rate of 0.375 Gy/min. The beam profile of the Pantak X-ray unit was in agreement with the ISO-4037:1996 requirements; the air kerma rate at each point of test never varied more than 5% over the entire sensitive area of the irradiated sample. Fetuses were isolated from the mother at E18, shortly before birth, to prevent fetal loss due to maternal cannibalism of the malformed pups. The mothers were killed by cervical dislocation, followed by surgical removal of both uterine horns and retrieval of the fetuses from the uterus and the amniotic sacs. The procedure was performed at room temperature. The fetuses were weighed and resorptions/lately dead fetuses were counted and classified as (1) "early resorptions" (fleshy placental mass with or without embryonic rudiments), (2) "resorptions with clear fetal structures," or (3) "lately dead fetuses" (fully formed but without signs of life). A subsequent categorical macroscopic scoring of various malformations, including microphthalmos/complete apparent anophthalmos (MA) and iris malformations, NTDs (e.g., exencephaly and spina bifida), cleft palate, gastroschisis, agnathia, and open eyelids was performed using a Leica stereo dissection microscope as previously described (Baatout et al., 2002; Bekaert et al., 2005; Jaramillo-Rangel et al., 2013). Although animals were macroscopically checked for spina bifida and coloboma of the iris, the prevalence was negligibly low, thus not meriting further discussion in this paper (0 cases of macroscopically detectable spina bifida in all groups and 1 case of keyhole-coloboma after 0.5 Gy at E7.5). EDs (MA or iris malformations) were quantified either exclusively in the left eye or the right eye, or both eyes simultaneously. The sum total of these three groups was calculated and depicted below as well, thus illustrating the sum total amount of fetuses with a form of ED. A more in-depth characterization of iris malformations was performed via histological analyses. Here for, the heads of animals that demonstrated iris malformations were fixed overnight in 4% paraformaldehyde and subsequently subjected to paraffin sectioning (7.00 μm slice thickness) in the horizontal plane, as well as hematoxylin and eosin staining. Next, sections were imaged using a bright-field Nikon Ti-Eclipse microscope. This experiment is illustrated in Table 1 (2.1).

TABLE 1 Tabular form of the performed experiments, covered in detail in the "2. Materials and methods" chapter

Study	Irradiation stage	Dose	Age of analysis	Type of analysis
chapter 2.1 and 2.2	E7, E7.5, E8, E8.5, E9	Control, 1.0 Gy	E18	Macroscopic assessment, Axial skeletal analysis, fetal weight, prenatal death
	E7.5	Control, 0.5 Gy	E18	
chapter 2.3	E7.5	Control, 0.1 Gy, 0.5 Gy, 1.0 Gy	W5	Survival analysis
		Control, 0.1 Gy, 0.5 Gy	W10	Body and brain weight

2.2 | Skeletal stainings

Skeletal stainings were performed using a modified alcian blue/alizarin red procedure, where the fetuses were heated in water (45 s, 70°C) and subsequently skinned, gutted and had their adipose tissue removed (Kimmel & Trammell, 1981) (Table 1, 2.2). Next, they were rinsed with phosphate buffered saline and stained for 42 hr. Next, a digestion was performed using 1% KOH (50–85 h) and specimens were stored in 1:1 H_2O:glycerol for 24 hr, which was followed by long term storage in 100% glycerol (Kimmel & Trammell, 1981). The skeletons were subjected to a qualitative analysis of general skull morphology using a Leica stereomicroscope.

2.3 | Postnatal viability

Continuing on the observations made in the macroscopic experiment, pregnant animals were irradiated with doses of 0.0 Gy (control), 0.1 Gy, 0.5 Gy, or 1.0 Gy at E7.5 and allowed to deliver the pups (Table 1, 2.3). 0.1 Gy and 0.5 Gy were included in these postnatal experiments to offer a more profound view on possible dose-dependent effects. Next, survival chances of the progeny were assessed at 5 weeks of age (W5). As a follow up, the surviving pups were grown until W10 after irradiation at E7.5 with 0.0 Gy (control), 0.1 Gy, or 0.5 Gy, and subsequently their body and brain weight were registered. The 1.0 Gy group was no longer included in this latter analysis, considering the outcome of the survival study at W5.

2.4 | Statistics

Statistical analyses of the macroscopic defects and prenatal deaths at E18 (individual phenotypes of early resorptions, fetal structures, and lately dead) were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons (with correction). For the count of conceptuses, the summarized data of the prenatal deaths, the fetal weights at E18 and the body and brain weights at W10, a one-way ANOVA with Dunnett's multiple comparisons (with correction) test was used. Analysis of the survival data was performed using the log-rank test, comparing the four survival curves (control, 0.1 Gy, 0.5 Gy, and 1.0 Gy). All analyses were performed using the Prism 7.02 Graphpad software. The litter was always used as the statistical sampling unit (Lazic & Essioux, 2013), except for the survival analysis at W5, where the number of individual pups were used as the statistical sampling unit.

3 | RESULTS

3.1 | Assessing embryonic radiosensitivity at various neurulation stages

3.1.1 | Macroscopic observations of congenital defects

Microphthalmos/anophthalmos (MA)

When taking into account the sum of all MA incidences at E18, we observed a significant increase in MA prevalence following 1.0 Gy X-rays at E7, E7.5 and E8.5, while the highest prevalence was observed after irradiation at E7.5 ($62.07 \pm 5.06\%$, Table 2: MA total). A significant increase in the number of fetuses that had both eyes affected simultaneously at E18 was observed in the E7.5 ($30.65 \pm 5.21\%$) and E8.5 ($14.17 \pm 5.46\%$) groups (Table 2: MA bilateral), all as compared to nonirradiated controls ($0.38 \pm 0.38\%$). Similar trends could be observed in animals that had only one eye with MA. Interesting however, the defect after irradiation with 1.0 Gy was more frequently observed in the right eye (E7 [$19.86 \pm 4.68\%$], E7.5 [$23.74 \pm 3.25\%$], control [$0.98 \pm 0.70\%$], Table 2: MA unilateral right eye) as compared to the left eye (E7.5 [$7.68 \pm 1.98\%$], control [$0.30 \pm 0.30\%$], Table 2: MA unilateral left eye).

Iris malformations

We noted a significant gain in the prevalence of all iris malformations after exposure with 1.0 Gy at E7 ($18.12 \pm 5.38\%$) (control: $0.76 \pm 0.53\%$) (Table 2: iris malformations total).

TABLE 2 Percentages of E18 fetuses displaying either MA (microphthalmos/anophthalmos), iris malformations, exencephaly, cleft palate, gastroschisis, agnathia or open eyelids after irradiation with 0.0 Gy or 1.0 Gy at E7, E7.5, E8, E8.5, or E9, and 0.5 Gy at E7.5

		Control	E7	E7.5		E8	E8.5	E9
		0.0 Gy	1.0 Gy	0.5 Gy	1.0 Gy	1.0 Gy	1.0 Gy	1.0 Gy
	***N* (litters)**	33	15	26	30	16	16	8
	***n* (fetuses)**	237	76	204	202	109	99	59
MA (total)	**Pups affected**	4	24	28	124	12	17	5
	Events/litter (%)	1.67	36.04	13.24	62.07	13.44	21.80	7.29
	SEM	0.83	8.91	2.58	5.06	3.77	6.59	4.84
	Adjusted *p* value	na	<.0001	.0334	<.0001	.1196	.0149	>.9999
MA (bilateral)	**Pups affected**	1	3	8	58	6	10	4
	Events/litter (%)	0.38	8.52	3.58	30.65	7.14	14.17	5.90
	SEM	0.38	6.66	1.39	5.21	3.41	5.46	3.87
	Adjusted *p* value	na	>.9999	>.9999	<.0001	.4342	.0274	>.9999
MA (unilateral right eye)	**Pups affected**	2	16	18	48	4	6	0
	Events/litter (%)	0.98	19.86	8.59	23.74	4.37	6.39	0.00
	SEM	0.70	4.68	2.06	3.25	2.29	2.44	0.00
	Adjusted *p* value	na	<.0001	.0325	<.0001	>.9999	.4505	>.9999
MA (unilateral left eye)	**Pups affected**	1	5	2	18	2	1	1
	Events/litter (%)	0.30	7.67	1.07	7.68	1.94	1.25	1.39
	SEM	0.30	5.14	0.76	1.98	1.33	1.25	1.39
	Adjusted *p* value	na	.5550	>.9999	<.0001	>.9999	>.9999	>.9999
Iris malformation (total)	**Pups affected**	2	16	6	8	4	3	3
	Events/litter (%)	0.76	18.12	3.08	3.18	3.47	3.76	6.25
	SEM	0.53	5.38	1.30	0.99	1.89	2.28	4.38
	Adjusted *p* value	na	.0002	>.9999	.5702	>.9999	>.9999	>.9999
Iris malformation (bilateral)	**Pups affected**	1	1	1	0	1	0	1
	Events/litter (%)	0.38	0.95	0.43	0.00	1.04	0.00	2.08
	SEM	0.38	0.95	0.43	0.00	1.04	0.00	2.08
	Adjusted *p* value	na	>.9999	>.9999	>.9999	>.9999	>.9999	>.9999
Iris malformation (unilateral right eye)	**Pups affected**	1	6	5	5	3	1	2
	Events/litter (%)	0.38	7.13	2.65	1.97	2.43	2.08	4.17
	SEM	0.38	2.42	1.10	0.82	1.68	2.08	4.17
	Adjusted *p* value	na	.0025	.5287	>.9999	>.9999	>.9999	>.9999

(Continues)

TABLE 2 (Continued)

		Control	E7	E7.5		E8	E8.5	E9
		0.0 Gy	1.0 Gy	0.5 Gy	1.0 Gy	1.0 Gy	1.0 Gy	1.0 Gy
Iris malformation (unilateral left eye)	**Pups affected**	0	9	0	3	0	2	0
	Events/litter (%)	0.00	10.04	0.00	1.22	0.00	1.67	0.00
	SEM	0.00	3.85	0.00	0.68	0.00	1.15	0.00
	Adjusted *p* value	na	<.0001	>.9999	.9151	>.9999	.7763	>.9999
Exencephaly	**Pups affected**	0	3	2	29	1	0	0
	Events/litter (%)	0.00	5.03	0.87	14.32	0.89	0.00	0.00
	SEM	0.00	3.41	0.60	3.12	0.89	0.00	0.00
	Adjusted *p* value	na	.5014	>.9999	<.0001	>.9999	>.9999	>.9999
Cleft palate	**Pups affected**	0	3	4	9	1	3	2
	Events/litter (%)	0.00	2.96	1.76	4.63	3.13	4.58	2.78
	SEM	0.00	1.61	0.83	1.40	3.13	3.15	1.82
	Adjusted *p* value	na	.4413	.7111	.0042	>.9999	>.9999	.7112
Gastroschisis	**Pups affected**	1	7	2	18	0	2	0
	Events/litter (%)	0.38	7.16	1.19	10.57	0.00	4.17	0.00
	SEM	0.38	3.09	0.83	3.62	0.00	2.85	0.00
	Adjusted *p* value	na	.0405	>.9999	.0003	>.9999	>.9999	>.9999
Agnathia	**Pups affected**	1	1	7	33	0	2	0
	Events/litter (%)	0.38	2.22	3.06	16.41	0.00	3.65	0.00
	SEM	0.38	2.22	1.47	3.44	0.00	2.52	0.00
	Adjusted *p* value	na	>.9999	.8893	<.0001	>.9999	>.9999	>.9999
Open eye (total)	**Pups affected**	0	1	6	7	12	3	3
	Events/litter (%)	0.00	1.33	2.86	3.86	9.47	2.60	6.60
	SEM	0.00	1.33	1.60	1.45	4.24	1.42	3.48
	Adjusted *p* value	na	>.9999	.7277	.0865	.0188	.6468	.0621
Open eye (bilateral)	**Pups affected**	0	0	1	0	1	0	0
	Events/litter (%)	0.00	0.00	0.48	0.00	0.69	0.00	0.00
	SEM	0.00	0.00	0.48	0.00	0.69	0.00	0.00
	Adjusted *p* value	na	>.9999	>.9999	>.9999	.4966	>.9999	>.9999
Open eye (unilateral right eye)	**Pups affected**	0	1	2	0	5	1	2
	Events/litter (%)	0.00	1.33	1.03	0.00	4.04	0.78	3.47
	SEM	0.00	1.33	0.72	0.00	2.33	0.78	2.33
	Adjusted *p* value	na	>.9999	>.9999	>.9999	.0605	>.9999	.0620

(Continues)

TABLE 2 (Continued)

		Control	E7	E7.5		E8	E8.5	E9
		0.0 Gy	1.0 Gy	0.5 Gy	1.0 Gy	1.0 Gy	1.0 Gy	1.0 Gy
Open eye (unilateral left eye)	**Pups affected**	0	0	3	7	6	2	1
	Events/litter (%)	0.00	0.00	1.35	3.86	4.74	1.82	3.13
	SEM	0.00	0.00	0.75	1.45	2.56	1.26	3.13
	Adjusted *p* value	na	>.9999	>.9999	.0226	.2238	>.9999	>.9999
All malformations	**Pups affected**	7	38	36	145	28	23	13
	Events/litter (%)	2.80	54.05	17.50	72.20	26.04	28.28	23.78
	SEM	1.25	7.05	2.83	4.39	4.48	7.70	6.13
	Adjusted *p* value	na	<.0001	.0274	<.0001	.0023	.0079	.0758

The highest incidence of MA could be observed after irradiation at E7.5. E7 proved to be most radiosensitive day for inciting iris malformations (total and unilateral right and left). Exencephaly, cleft palate and agnathia were also most strongly induced with 1.0 Gy at E7.5, whereas for gastroschisis it was E7. Open eye (total) was most frequently induced following 1.0 Gy irradiation at E8, whilst unilateral left open eye was most radiosensitive at E7.5.

However, we found no difference in the frequency of iris malformations affecting both eyes simultaneously after prenatal irradiation with 1.0 Gy (Table 2: iris malformations bilateral). In contrast, prevalence of iris malformations affecting only the right (Table 2: iris malformations unilateral right eye) or only the left (Table 2: iris malformations unilateral left eye) eye were shown to strongly increase after irradiation with 1.0 Gy at E7 (right: 7.13 ± 2.42%, left: 10.04 ± 3.85%, right control: 0.38 ± 0.38%, left control: 0.00 ± 0.00%). To closer investigate these malformed eyes, histologically stained sections of E18 fetuses irradiated with 1.0 Gy at E7.5 were examined next. In control fetuses at E18 the so-called lens stalk has already disappeared through carefully programmed apoptosis (Ozeki et al., 2001) (Figure 1a), whereas we could demonstrate the persistent presence of a lens stalk and a reduced lens size at E18 in the eyes of irradiated animals with the iris malformation phenotype (Figure 1b, black arrow). These findings thus support a possible delay in eye development and suggest that the defect may be classified in the family of congenital lens opacities (Smith, Roderick, & Sundberg, 1994).

Exencephaly

Prevalence of exencephaly was significantly increased in E7.5 (14.32 ± 3.12%) groups irradiated with a dose of 1.0 Gy, as compared to controls (0.00 ± 0.00%) (Table 2: exencephaly).

Other malformations

A dose of 1.0 Gy at E7.5 resulted in a significant rise of cleft palate (4.63 ± 1.40%), as compared to nonirradiated controls (0.00 ± 0.00%) (Table 2: cleft palate). The rate of gastroschisis cases was significantly augmented by irradiation with 1.0 Gy at E7 (7.16 ± 3.09%) and E7.5 (10.57 ± 3.62%) (control: 0.38 ± 0.38%) (Table 2: gastroschisis). Malformations of the jaw (agnathia) were significantly more prevalent in the E7.5 group (16.41 ± 3.44%) (control: 0.38 ± 0.38%) (Table 2: agnathia). Finally, the total incidence of open eye increased

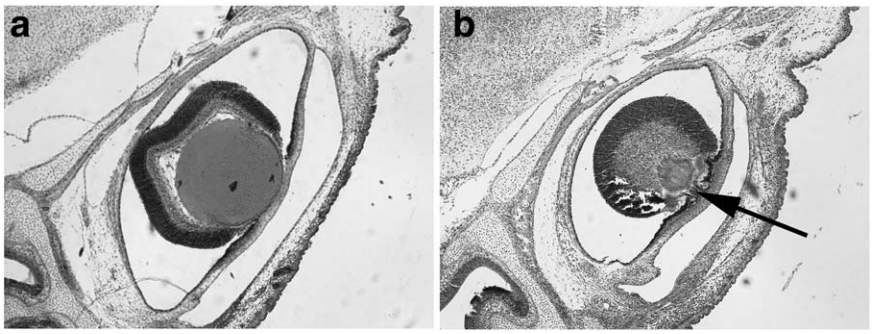


FIGURE 1 H&E stainings of E18 fetal eyes with iris malformations following prenatal irradiation during neurulation. Histological analysis of the eyes at E18 show that the control animal had lost its lens stalk as was previously described (a), whereas eyes with iris malformations still had an intact lens stalk (b, black arrow), a structure normally lost to apoptosis during late neurulation

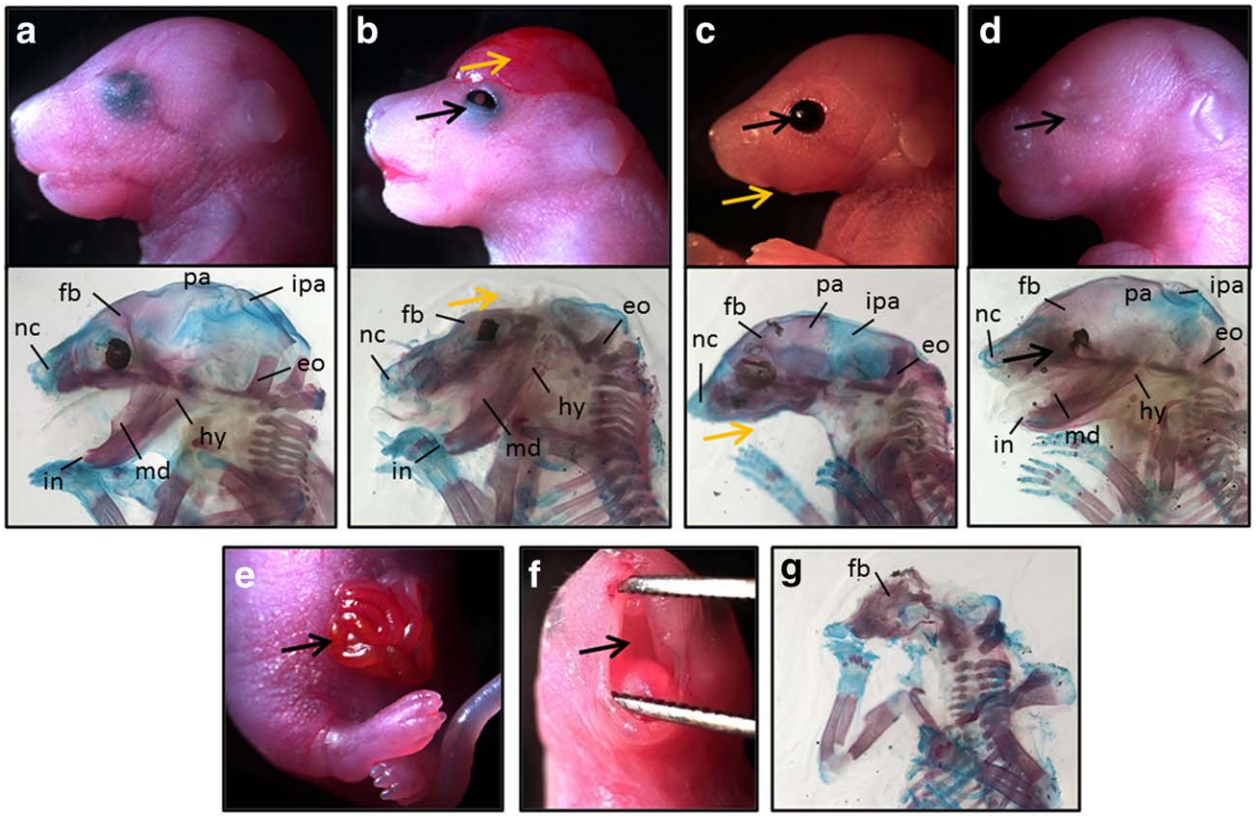


FIGURE 2 Examples of various congenital defects observed at E18 after prenatal irradiation during neurulation with 1.0 Gy of X-rays and representative alcian blue/alizarin red dual stainings of the respective anomalies. (a) normal control embryo. (b) irradiated embryo with exencephaly (orange arrow) and open eye (black arrow). (c) irradiated embryo with agnathia (orange arrow), open eye and iris malformation (black arrow). (d) irradiated embryo with MA (black arrow). (e) gastroschisis on an irradiated embryo (black arrow). (f) an irradiated embryo with cleft palate (black arrow). (g) combined exencephaly/agnathia/MA after irradiation. The frontal bone (fb), parietal (pa), interparietal (ipa), exoccipital (eo), incisors (in), mandible (md), hyoid (hy), and nasal cavity (nc) are indicated in the figure

significantly after 1.0 Gy at E8 (9.47 ± 4.24%) (Table 2: open eye total). More specifically, unilateral left open eye increased significantly for irradiation at E7.5 (3.86 ± 1.45%) (Table 2: open eye unilateral left), whereas there were no increases observed for open eye affecting solely the right eye, nor bilateral open eye (control: 0.00 ± 0.00%). Although whole body examinations were performed, no observations of other NTDs such as spina bifida were made in neither control nor irradiated animals (data not shown).

Total malformations

When looking at the accumulated total of all fetuses affected by at least one congenital defect after irradiation with 1.0 Gy, a significant increase was detected in all irradiated groups, except E9, as compared to the control group (Table 2: all malformations). Notably, the strongest increase was observed after irradiation at E7.5 (72.20 ± 4.39%), as compared to nonirradiated controls (2.80 ± 1.25%) (Table 2: all malformations). A similar trend was observed in regard to animals affected by two or more defects concurrently (data not shown), suggesting that phenotypical severity was worst at E7.5.

Because the highest prevalence of total malformations was observed at E7.5, an additional cohort of mice was irradiated with 0.5 Gy at this specific stage, this to unveil possible dose-dependent effects. For the MA phenotypes, we still observed a significant increase in total MA incidence (13.24 ± 2.58%), and in unilateral right eye MA (8.59 ± 2.06%), all as compared to controls (Table 2: MA). However, exposure to this lower dose of 0.5 Gy did not result in a significant increase of any of the other defects discussed above. In general, our data show an apparent dose response, with 0.5 Gy always resulting in lower prevalences than 1.0 Gy.

3.1.2 | Assessment of skeletal malformations

To offer a more structural understanding of the congenital anomalies observed after E7.5 X-irradiation with a dose of 1.0 Gy, a qualitative analysis of the skeletons was performed at E18 (see Figure 2a for a control, nonirradiated animal). In case of an exencephalic fetus, we found large parts of both ossified bone and cartilage to be missing, resulting in an

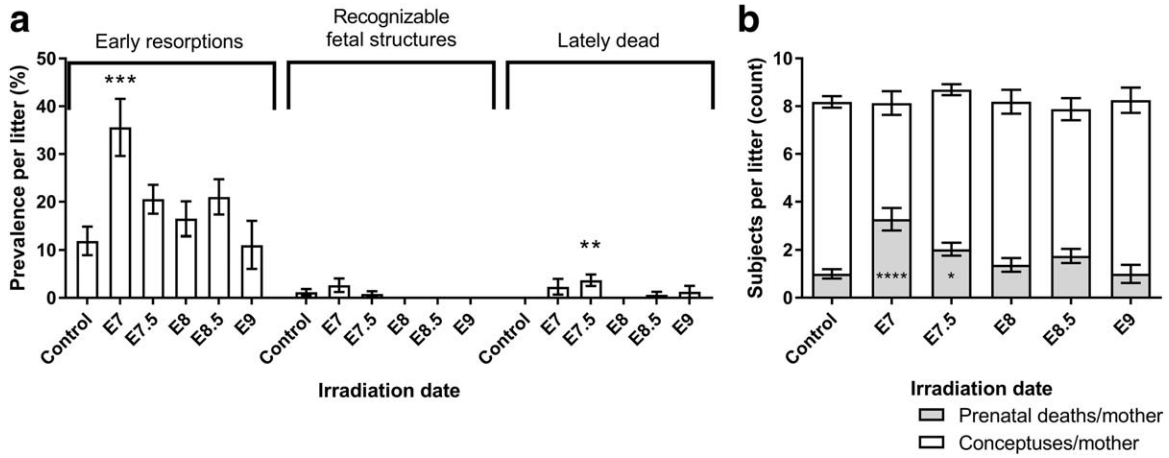


FIGURE 3 (a) Frequencies of early resorptions, resorptions with recognizable fetal structures and lately dead fetuses at E18 after prenatal irradiation at E7 (litters [N] $\geq$ 15, conceptuses [n] $\geq$ 122), E7.5 ($N \geq 30$, $n \geq 254$), E8 ($N \geq 16$, $n \geq 131$), E8.5 ($N \geq 16$, $n \geq 126$), E9 ($N \geq 8$, $n \geq 66$), or control ($N \geq 33$, $n \geq 270$). The most significant increases were observed after irradiation at E7 for the early resorptions and at E7.5 for the lately dead fetuses. (b) The amount of total conceptuses (prenatal deaths plus living fetuses) per mother at E18 was not altered by prenatal radiation exposure at E7, E7.5, E8, E8.5, or E9 with 1.0 Gy, whereas significant increases were present in the total number of prenatal deaths per mother after irradiation at E7 and E7.5. Error bars illustrate SEM. *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$

open skull vault. Indeed, areas of the frontal bone (fb), parietal (pa), and interparietal (ipa) skull appeared to be absent and also the orientation of the exoccipital (eo) bones were found to be abnormal (Figure 2b, orange arrow). Animals with agnathia clearly showed a completely absent jaw, with no incisors (in), mandible (md) nor hyoid (hy) present (Figure 2c, orange arrow). For fetuses displaying the MA phenotype, we generally observed no major skull malformations at the height of the eye sockets. However, the notion of a reduced eye size and a reduced presence of retinal pigment epithelium (Figure 2d) supports the classification as MA. Gastroschisis, with a distinct lack of peritoneum material (Figure 2e), as well as cleft palate featuring a complete split (Figure 2f), could clearly be observed macroscopically. Finally, fetuses displaying a combined phenotype of, for example, exencephaly, MA and agnathia, demonstrated severe hypoplasia of the skull with hallmark structures such as the nasal cavity (nc), pa, ipa, eo, in, md, and hv missing (Figure 2g).

3.1.3 | Assessment of prenatal mortality

To reveal whether and how irradiation during neurulation affects prenatal mortality, animals were exposed in utero to 1.0 Gy at different stages of neurulation. Irradiation at E7 resulted in a significantly increased number of early resorptions (35.59 ± 5.99%) in comparison to controls (11.88 ± 2.94%) (Figure 3a). The number of resorptions with recognizable fetal structures also seemed to be higher in the E7 group, although data did not reach significance. Finally, X-ray exposure at E7.5 resulted in a significant higher frequency of lately dead fetuses (3.68 ± 1.22%, control: 0.00 ± 0.00%). Of note, irradiation with 0.5 Gy at E7.5 did not cause any significant alteration in the number of prenatal deaths (early resorptions = 9.02 ± 1.91%, recognizable fetal structures = 0.38 ± 0.38%, lately dead = 0.78 ± 0.54%) as compared to controls (respectively 11.88 ± 2.94%, 7.94e-18 ± 7.94e-18% and 0.00 ± 0.00%). The irradiated pregnant females themselves did not suffer from increased mortality following irradiation (0 females died during pregnancy following irradiation) and the total number of conceptuses (prenatal deaths plus living fetuses) per mother did not change after irradiation with 1.0 Gy (Figure 3b). In addition, the total number of prenatal deaths per mother at E18 significantly increased after irradiation (1.0 Gy) at E7 and E7.5

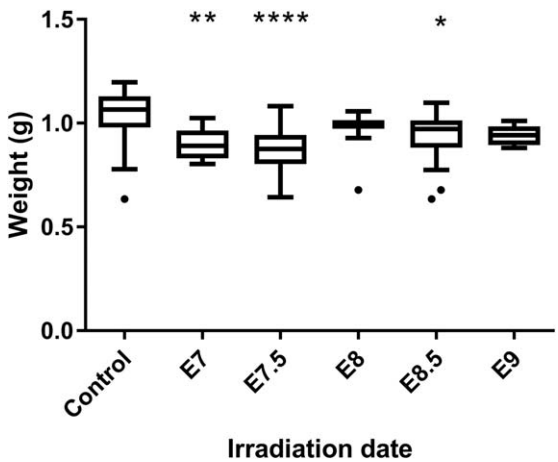
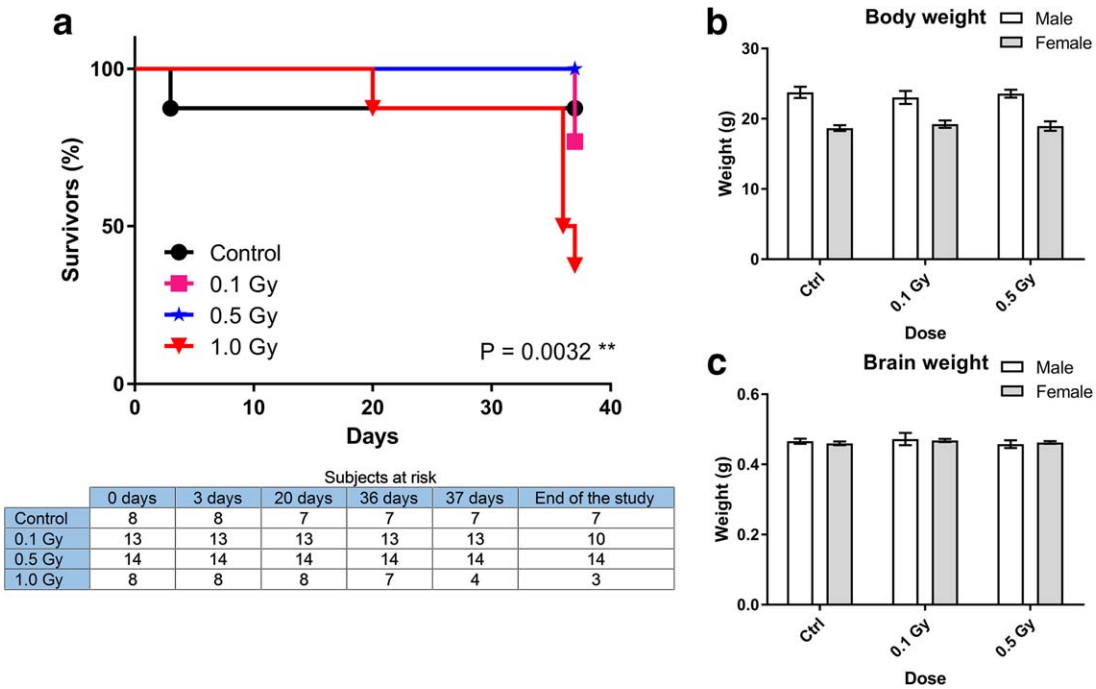


FIGURE 4 Embryonic weights at E18 after irradiation at E7 (litters [N] $\geq$15, embryos [n] $\geq$73), E7.5 ($N \geq 30$, $n \geq 193$), E8 ($N \geq 16$, $n \geq 109$), E8.5 ($N \geq 16$, $n \geq 98$), E9 ($N \geq 8$, $n \geq 58$), or control ($N \geq 33$, $n \geq 237$). Significant decreases were observed after irradiation with 1.0 Gy at E7, E7.5, and E8.5. *$p \leq .05$, **$p \leq .01$, ****$p \leq .0001$



Subjects at risk

	0 days	3 days	20 days	36 days	37 days	End of the study
Control	8	8	7	7	7	7
0.1 Gy	13	13	13	13	13	10
0.5 Gy	14	14	14	14	14	14
1.0 Gy	8	8	8	7	4	3

FIGURE 5 Postnatal survival and adult body/brain weight after irradiation at E7.5. (a) Kaplan-Meier survival graph after irradiation at E7.5 with either 0.0 Gy (control), 0.1 Gy, 0.5 Gy, or 1.0 Gy. Log-rank testing suggested a significant decrease of survival by W5 ($N \geq 2, n \geq 8$). The table of subjects at risk illustrates the absolute number of pups at risk at the start of each experimental day. Body weights (b) and brain weights (c) after prenatal radiation exposure at E7.5 (control, 0.1 Gy, or 0.5 Gy) show no significant changes with these doses ($N \geq 4, n \geq 7$). $^{**}p \leq .01$

(Figure 3b), supporting the findings in (Figure 3a). A lower dose of 0.5 Gy at E7.5 had no impact on the number of conceptuses and prenatal deaths per mother.

3.1.4 | Fetal weights

In addition to categorizing the macroscopic phenotypes of the irradiated animals, E7.5 irradiated fetuses were weighed at E18 to assess general growth retardation (Figure 4). Fetal weights at E18 were significantly decreased in all 1.0 Gy irradiated groups, except E8. No significant weight loss was observed using a lower dose of 0.5 Gy at E7.5 (data not shown).

3.2 | Postnatal viability after irradiation at E7.5

Considering the major impact of irradiation at E7.5 on embryonic exencephaly and MA development, we ventured to also assess postnatal and adult viability after irradiation at E7.5. As is portrayed by the Kaplan-Meier survival graph (Figure 5a), by W5, we observed a significantly reduced postnatal survival following prenatal X-ray exposure as compared to the controls (12.50% mortality). The highest mortality was observed after 1.0 Gy (62.50% mortality). The marked incidence of death at days 36 and 37 following prenatal 1.0-Gy irradiation may be linked to severe hemorrhaging in the brain, as intracranial bleeding and hydrocephalus was observed in all animals that died at days 36 and 37. Next to mortality, we also documented body and brain weights of mice at W10, following irradiation with 0.1 Gy or 0.5 Gy at E7.5. Of note, 1.0 Gy irradiated animals were excluded from this experiment, given the high mortality in this group. No differences in male and female body and brain weights were observed after irradiation with 0.5 Gy or lower (Figure 5b,c).

4 | DISCUSSION

Epidemiological studies performed on survivors of the Chernobyl disaster have previously suggested that cranial NTDs and EDs in humans may be linked to prenatal radiation exposure. Thus, our study focused on the effects of irradiation during the neurulation period, during which the foundation of the CNS (including the eyes) is formed and of which little is known in terms of radiosensitivity. This developmental period, which in mice occurs approximately from E7.5 to E9, starts at the neural plate, and encompasses neural plate invagination, neural fold fusion and finally formation of the NT. Our findings confirmed that exposure to ionizing radiation during neurulation resulted in NTDs and EDs, as was also suggested by others (Baatout et al., 2002; Di Majo, Ballardin, & Metalli, 1981), and expanded on this knowledge through a more detailed analysis on the most radiosensitive stage.

Although several reports over the past years have discussed the impact of prenatal irradiation on embryogenesis, knowledge on the effects of radiation exposure at specific stages of neurulation is limited. Several of these studies have previously identified the overall neurulation period as being highly radiosensitive, however these studies often had limitations in terms of long coupling times, long time intervals between developmental stages at which irradiation was performed and a lack of statistical power (Russell, 1950, 1956). It should be noted that the neurulation period is a very dynamic phase of embryogenesis and consists of many cellular and molecular events, which implies that the embryo has very distinct and unique characteristics depending on the (hour of the) developmental day (Yamaguchi & Miura, 2013), reinforcing the importance of clearly defining the developmental stage at X-ray exposure.

Previous studies have to a certain extent investigated the radiosensitivity and underlying mechanisms behind both preneurulation and postneurulation development (e.g., neurogenesis) (Quintens et al., 2015; Verreet et al., 2015), but knowledge on the mechanisms underlying radiosensitivity during neurulation is limited at best. In addition, differences in irradiation conditions, mouse strain dependency (Streffer & Müller, 1996) and variations in developmental stages make it difficult to form a unified picture of the phenotypes resulting from in utero irradiation during mid-gestation. For subsequent future mechanistic studies, it is thus of crucial importance that neurulation radiosensitivity is better defined and with lower doses ($\leq$1.0 Gy).

4.1 | Macroscopic study

Macroscopic evaluations of E18 fetuses irradiated during neurulation revealed a number of congenital malformations that will be discussed below. In this work many of the fetuses irradiated at E7.5 demonstrated more than one malformation, which may be the consequence of several (possibly independent) developmental processes being affected. This finding is in line with a previous study in which pregnant mice were exposed to X-rays at E7.5, showing multiple defects in the exposed fetuses. For example, exencephaly and anophthalmia often coincided with malformations of the jaw (Di Majo et al., 1981).

Exencephaly which is believed to be comparable to anencephaly in humans (Timor-Tritsch, Greenebaum, Monteagudo, & Baxi, 1996) was most frequently induced after E7.5 irradiation. This finding is in contrast to a study of Russel, who described no statistically proven link between exencephaly and irradiation during neurulation, but is in line with another study that also identified E7.5 as being more prone to radiation-induced exencephaly. Yet, since exencephaly is thought to primarily arise from anomalous NT closure, which can be observed around E9 (Copp, 2005; Yamaguchi & Miura, 2013), the heightened risk to develop exencephaly after E7.5 irradiation was unexpected (Di Majo et al., 1981). A number of hypotheses can be put forward to explain this high radiosensitivity after E7.5 irradiation. First, a significant apoptotic response has been observed previously at 6 hr after irradiation at E6.5–E7.5, which dropped back to basal levels at 12–24 hr postirradiation (Heyer, MacAuley, Behrendtsen, & Werb, 2000). The observed massive apoptosis might indicate early depletion of key cells that give rise to crucial structures during neurulation. Of note, however, induction of apoptosis after irradiation at other stages of neurulation has not been studied in great detail so far, but would improve our understanding of varying radiosensitivity between developmental stages.

We also observed an increased incidence of MA in fetuses irradiated during neurulation. This is in line with previous descriptive studies (Di Majo et al., 1981; Russell, 1950, 1956). Gamma-irradiation with a dose of 0.75 Gy at E9 did not elicit a significant increase in malformations, whereas 0.1 Gy of heavy ions (Ar, Fe) at E9 induced MA at E18 (Hirobe, Eguchi-Kasai, Sugaya, & Murakami, 2013). Little is known about detailed differences in radiosensitivity throughout the neurulation period with X-ray doses $\leq$ 1.0 Gy. Hereto, our group is the first to identify E7.5 as the most radiosensitive neurulation stage with such doses. Interestingly, as for exencephaly, eye development starts approximately at E8.5 (in parallel to early NT closure) and continues until after birth. Formation of most major structures of the eye, that is, the detached lens and the rudimentary retina, can be observed at approximately E10.5–E11.5 (Parvini et al., 2014). Thus, to explain the time-lapse between radiation-induced damage at E7.5 and the affected developmental processes, it is important to assess the underlying mechanisms and molecular key players, which remain largely elusive. Strikingly, from our data, there appears to be a bias towards the development of right eye MA after irradiation, as compared to the prevalences seen for the left eye. This might be explained by the fact that our mouse strain (C57BL6/J) is known to have a strong tendency to spontaneously develop asymmetrical MA, with a higher prevalence of right eye MA. Of note, a review by R. S. Smith et al. (1994) stated that the tendency of the C57BL strain to develop asymmetrical eye defects may not be the result of a specific mutation, but that baseline genetic background may facilitate the possibility of developing such congenital defects more easily under the influence of external factors. The precise underlying genetic factors and their associated mechanisms have, however, not yet been defined. Caution must thus be taken in assuming that X-irradiation can by itself skew the MA phenotype towards the right eye. In turn, it may be of interest to assess radiosensitivity during neurulation using a different mouse strain that does not show this genetic predisposition. In any case, having identified E7.5 as the most

radiosensitive stage for eliciting MA has paved the way for future mechanistic studies.

The highest incidence of iris malformations occurred after irradiation with 1.0 Gy at E7. An older study showed that irradiation of C3H mice during neurulation (at E9), using a dose of 1.75 Gy, increased the prevalence of iris/retinal malformations by 29.5% (Cekan, Tribukait, & Vokal-Borek, 1985). In contrast, we observed a small but insignificant increase at E9, but the large effect observed by Cekan et al. may be the result of the higher dose that was used. In our study, histological analyses of the eyes of prenatally irradiated animals revealed an intact lens stalk, which has previously been observed in the birth defect termed "congenital lens opacity" in C57BL6/J mice (Smith et al., 1994). How this defect may relate to other radiation-induced EDs, such as cataract (Auvinen, Kivelä, Heinävaara, & Mrena, 2015; Hammer et al., 2013) (also classified as a "lens opacity") remains to be elucidated. Of note is that cataract can be congenital in nature as well (Deng & Yuan, 2014; Pichi, Lembo, Serafino, & Nucci, 2016), although a link between prenatal radiation exposure and the incidence of this congenital cataract is debatable (Lie et al., 1992). Of interest, reduced lens stalk apoptosis is known to lead to iris malformations that closely resemble our radiation-induced phenotype. Surprisingly, however, we observed the iris malformations primarily after irradiation at E7, whereas lens stalk apoptosis does not start until E11 and lasts until E12 (Ozeki et al., 2001). This suggests that other mechanisms are responsible for the radiation-induced iris malformations, especially since a decreased apoptosis after irradiation is unlikely. In turn, it may be the result of a general delay in development. In all, the underlying mechanisms of how irradiation at E7 can induce iris malformations may be multifold and elusive in nature, reinforcing the need for future research.

Finally, other defects apart from NTDs and EDs were found after irradiation during the neurulation period, in agreement with previous observations by other groups. A publication by S.H. Kim et al. reported that high dose (2.0 Gy, 10 Gy/min) gamma irradiation of pregnant ICR mice during discrete stages of development induced the highest incidence of gastroschisis after irradiation at E7.5 (2.22%), which is in accordance with our results obtained with X-rays. Interestingly, in their study the most radiosensitive stage for developing cleft palate was identified as E11.5 (36.59%), whereas we observed the highest incidence at E7.5 (Kim et al., 2001). Continuing, in swiss albino mice irradiated with 0.5 Gy Gamma rays, open eye defects were also found to be significantly more prevalent after irradiation at E17, suggesting that the induction of both cleft palate and open eye defects is not limited to irradiation during neurulation (Hossain, Devi, & Bisht, 1999). Even though our results indicate that E8 is the most radiosensitive stage for inducing open eyelids, it may in turn be of interest for future studies to assess postneurulation sensitivity. Indeed, in terms of eyelid development, the epithelial ridges that will give rise to the eyelids normally meet and fuse at E16.5, implying that failure to initiate eyelid formation or failed fusion may also lead to the open eyelid phenotype (Gelineau-van Waes et al., 2008). Yet, how irradiation during very early development (neurulation) induces the open eyelid phenotype, remains to be elucidated.

Although the range of doses that was used in this study is limited, an apparent dose response for the induction of congenital anomalies was visible after irradiation with 0.5 Gy and 1.0 Gy. The dose threshold (if there exists one) does not appear to be the same for every observed malformation, with unilateral right MA (and in turn total MA) being significantly increased following 0.5 Gy, while no other defect was significant increased at this dose. Instead, all other defects required a dose of 1.0 Gy for a significant increase in prevalence.

In general, we can conclude that irradiation during early neurulation (E7.5) appears to affect developmental processes that manifest only several days after initial radiation exposure. The exact causes remain to be elucidated and offer interesting research opportunities that may address the mechanisms behind radiation exposure and neurulation in general. Interesting leads that can be pursued may include the identification of radiosensitive progenitor cell subsets early after irradiation and persistently altered gene expression during NT closure and eye development.

4.2 | Prenatal mortality and fetal weight

Prenatal death caused by ionizing radiation has been studied extensively and an all-or-nothing principle has been proposed to occur primarily during the pre-implantation phase. Similar as in our findings, a study by V. Di Majo revealed an increase in the rate of resorptions in (C57BL/Cne X C3H/Cne)F1 mice X-irradiated at E7.5. Their study, however, did not differentiate between early and late resorptions. Their results demonstrated a higher prevalence of resorptions ranging from 2.6% in the control group to 6.0% in the 1.0 Gy irradiated group. This frequency increased even further in the 2.0 Gy group, with a maximum reaching 74.3%. Notably, our data demonstrated a stronger significant increase in resorptions after E7.5 irradiation with 1.0 Gy, possibly due to strain differences (Di Majo et al., 1981), although detailed comparative studies are lacking. Our findings do support the study by S.H. Kim et al., who observed an elevated number of resorptions in the E7.5 group after exposure to gamma rays (2 Gy) in ICR mice (Kim et al., 2001). Dose-dependent fetal weight loss at E18 was reported by Di Majo as well, following exposure to X-irradiation (0.0–2.0 Gy) at the neurulation stage (E7.5), thus supporting our data (Di Majo et al., 1981). Continuing, a significant loss of body weight at

E18 was evidenced in several studies after exposure of ≥0.5 Gy of gamma rays at different stages of development (E6.5, E9, E11.5, and E17), which is in line with our findings as well (Bang et al., 2002; Hirobe et al., 2013; Hossain et al., 1999; Prakash Hande, Ume Devi, & Jagetia, 1990). It is thus important to note that the aforementioned prenatal weight loss could be considered a hallmark of irradiation throughout many stages of development.

4.3 | Postnatal mortality and adult weight

Postnatal mortality is known to already increase after exposure to 9.0 mGy of X-rays at E6.5 (preneurulation) in swiss albino mice (Prakash Hande et al., 1990). Interestingly, irradiation at only one developmental day later (E7.5) requires a high dose of 1.0 Gy, again demonstrating the difference in radiosensitivity at each developmental day. Information on adult weight after irradiation during neurulation is very sparse. Although we did not observe any significant weight loss after X-irradiation at E7.5, a recent study illustrated that irradiation at E11 with 0.66 Gy and 1.0 Gy, immediately after neurulation (i.e., at the time of neurogenesis), significantly reduced brain and body weights respectively at W50 (Verreet, Rangarajan, et al., 2016). Irradiation during even later stages of development (E14 and E17) using a ^{60}Co gamma source also showed decreased brain weight at 6 and 12 months of age, using doses starting at 0.5 Gy (Hossain, Chetana, & Devi, 2005). These data suggest that X-irradiation at high doses after neurulation may result in stunted brain and body weight, whereas irradiation at earlier stages has no clear persistent impact on adult weight.

5 | CONCLUSIONS

Our study shows that in C57BL6/J mice, E7.5 is the most radiosensitive stage for eliciting congenital defects such as MA and exencephaly, as well as several other malformations after exposure to doses of 0.5 Gy or 1.0 Gy. Early resorptions were most prominent after irradiation at E7, while the E7.5 stage appeared most sensitive for occurrence of late fetal deaths. In addition, irradiation with 1.0 Gy during most neurulation stages resulted in reduced fetal weights at E18. Finally, postnatal survival by W5 was significantly affected after 1.0 Gy irradiation at E7.5.

In all, our study offered a more detailed look into the effects of X-irradiation during neurulation and cautions against maternal ionizing radiation exposure during neural tube and eye development. Although epidemiological studies following the Chernobyl disaster suggest a link between in utero irradiation and NTDs and EDs, accurate dose estimations are often lacking. In addition, no such link was previously suggested following the A-bombings. Why this discrepancy exists remains unclear, but merits further research. In addition, the lack of data within the low-dose range has led to discussions as to whether congenital defects such as mental retardation follow a dose threshold model (Otake, Schull, & Lee, 1996), or a nonthreshold model (Osei & Darko, 2013). It is of interest for future studies to investigate these aspects in more detail, for all radiation-induced defects and in particular NTDs and EDs. Furthermore, in light of todays' global concern for radiation exposure following nuclear disasters or clinical procedures, it would be of interest for the radiation protection field to investigate possible antiteratogens for radiation-induced defects, for which our model of E7.5 irradiation would be highly suitable. Last, our model can serve to establish the molecular mechanisms that underlie radiation-induced NTDs and EDs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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