Development of zebrafish embryo model, an alternative *in vivo* vertebrate system for radiation biology research

Summary of Ph.D. Thesis

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I. INTRODUCTION

Radiation therapy is one of the most common methods to disrupt the ability of cancer cells to grow and divide to deprive of their multiplication potential in clinical applications despite having harmful effects on healthy tissues. Ionizing radiation (IR) is successfully used in patients with various primary and metastatic tumors (Larouche et al., 2007). More than 50% of all cancer patients are subject to radiotherapy during the course of their illness with an estimation that radiotherapy contributes to approximately 60% towards curative treatment (Baskar et al., 2012). Photon beam therapy is frequently used in the locoregional treatment of malignant tumor, it has also detrimental effects, with the aim of damaging the DNA of the cancerous cells and can also induce carcinogenesis in the surrounding healthy tissue of the tumor. Based on several decades of research on radiation biology, we have learned a lot about the pathomechanism and effects of ionizing radiation, which has led to a significant improvement in effective cancer cell killing and the preservation of normal tissue function, resulting in refinement in therapeutic methods.

Advanced photon delivery techniques with enhanced conformity and the rapidly growing installations of superconducting cyclotron/synchrotron-based particle therapy facilities have made hadron therapy available for an increasing number of cancer patients (Specht et al., 2015). Charged particle therapy leads to an increase in dose precision due to the energy deposition characterized by the Bragg peak. There is a growing worldwide interest in high linear energy transfer therapy with new hadron therapy centers and the radiobiology data generated by neutron therapy could help to develop novel in vivo model system for investigation of the biological effects and to develop biologically guided treatment approaches (Jones et al., 2001). During the last years, the more widespread application and the increasing number of patients and long-time survivors treated with proton therapy give rise to discussions on the biological response to proton radiation (Lühr et al., 2018).

The biological properties of any type of radiation are derived from the energy deposition pattern, which defines the molecular changes, in particular DNA damage and potential repair. It is therefore essential to study the biological effects of the different ionizing radiation qualities and combined approaches to precondition the safe clinical applications.

1.1. Radiation induced damages on normal tissue

The cellular damage caused by ionizing radiation may vary depending on the cell type, proliferation, intracellular and microenvironmental factors as well as the type of radiation, dose, fractionation and the radiation conditions (Blank et al., 1997). The ionizing radiation ionizes the atoms in the tissue it is travelling through, directly interact with cellular DNA and cause damage. The electrons ejected by
ionization can either straight act on the target or through the formation of free radicals, mainly from the radiolysis of water, producing subsequently reactive oxygen species and reactive nitrogen species which can attack cell membranes or break chemical bonds in biological molecules, leading to oxidative stress or DNA damage, termed indirect effect (Hurem et al., 2017). Whether the direct or indirect action is dominating, depends mainly on the linear energy transfer (LET) of the radiation, meaning the loss in energy of a charged particle per unit length of path of the medium it is travelling through. Therefore, low LET radiation, such as X- or γ-rays, rather acts by free radicals whereas high LET radiation, like neutrons or α-particles, which obviously have a higher biological effectivity, mainly damages the tissue by direct means (Prasad, 1995).

I.2. Therapeutic index

The therapeutic ratio indicates the association between the probability of tumor control and the likelihood of normal tissue damage. Improved therapeutic ratio represents a more favorable compromise between tumor control and toxicity (Zindler et al., 2015). There are several ways to increase the therapeutic index, the ratio between therapeutic effect and damage of healthy tissue: prolongation of treatment time, hyperfractionation or the use of radio-sensitizers and radio-protectors which specifically increase the sensitivity of tumor cells (Prasanna et al., 2012). In the case of cancer therapy, both the selective tumor cell sensitizer and normal tissue radio-protective agent could enhance the efficacy of radiotherapy. During the radiation therapy the normal tissue protection is essential in order to improve the therapeutic ratio.

I.3. Radiation modifiers

The use of radiation modifying agents allows to test the sensitivity of a biology system to detect differences in radiation effect. Furthermore, there is a great interest in developing radio-protectors to prevent normal tissue toxicity during chemo- and/or radiotherapy or nuclear accidents, for that reason, we investigated the effects of a potential radio-protector agent.

I.3.1. Potential therapeutic effects of phosphatidylcholine

Beneficial effects of dietary phospholipids have been known in relation to different illness and symptoms, including inflammation. Phosphatidylcholine (PC) is an essential component of endogenous surface-coating substance and biomembranes, and it is well founded that it is key lipid component of all kind of cell membranes and blood proteins (Volinsky and Kinunnen, 2013). L-alpha glycerylphosphorylcholine (GPC) is a water-soluble, deacylated PC intermediate which may be hydrolysed to choline and has been developed and studied as a potent anti-inflammatory and neuro-protective agent (Gallazzini and Burg, 2009). GPC has been proven to protect membranes from oxidation
and is able to improve membrane function after traumatic damage (Onishchenko et al., 2008). GPC has been exhibited protective activity in different experimental models against damage occurring due to inflammatory reaction (Tőkés et al., 2015).

I.4. Different radiation qualities

1.4.1. Neutron irradiation

Neutrons are a member of high LET radiation and therefore possess a higher biological effectivity, however, also have the potential to cause more damage in healthy tissues. Not only their energy is higher, neutrons also distribute in a much more precise way in the target tissue, all their energy concentrates on a few paths as compared to γ-rays whose energy is much more spread out.

1.4.2. Proton irradiation

Proton radiotherapy (PT) is hadrontherapy’s fastest growing method and the pillar of the fight against cancer which has rapidly evolved from the pioneering trials at the end of the 20th century to become an accepted alternative to conventional external beam radiotherapy with photons. These particles have shown to represent a high impact on the improvement of cancer therapy, mainly because of their preciseness of dose delivery selectively to the cancer (Levin et al., 2005). Due to this advantageous property, the tumor volume can be very precisely targeted with higher dose deposition at the end of the proton track, in the Bragg peak, means that PT offers the possibility of sufficient dose delivery to the tumor whilst simultaneously sparing the surrounding normal tissue (Hall and Giaccia, 2006).

1.5. Zebrafish as a model system

Zebrafish (Danio rerio) embryos have recently been introduced as a widely used novel vertebrate preclinical research model (Geiger et al., 2006). Their genomes share major homology with the human genome (70%), making them amenable for the study of various human diseases (Daroczi et al., 2006). This vertebrate model is an ideal test system as they have many positive attributes including high reproduction capacity and easy laboratory care at a relatively low cost. Embryo development is extremely rapid during the first few days post-fertilization whilst the embryos and larvae are transparent, giving the possibility to study the \textit{in vivo} organ development and perturbations after ionizing radiation (Bailey et al., 2009). With an irradiation size of about 1 mm, between cell monolayer culture and subcutaneous tumors or normal tissue organs in small animals (mice, rat), the zebrafish embryo could potentially be deployed for detailed investigations on the relative biological effectiveness (RBE) LET correlation and to quantify the low LET and high LET radiation induced damages at the total organism, at organ and at tissue level.
I.6. Biological model development for research on laser driven proton sources

The installation of ultrafast, high-energy lasers opens the possibility for development of innovative approaches in radiation oncology. There has been a vast development of laser-driven particle acceleration (LDPA) using high power lasers, resulting in short particle pulses of ultrahigh dose rate. At the actual status of the development, low energy, limited size beams are available under technical conditions for radiobiology experiments, therefore the zebrafish embryo as a small vertebrate model for comparative radiobiology studies can be used for laser-driven proton irradiation.

II. AIMS

The aims of our studies were:

a) establishment of optimal parameters for validation of zebrafish embryo model, definition of embryonal stages, doses and observational endpoints;
b) to develop a precise dose delivery technique and setup for reproducible irradiation;
c) to define the radiation dose-effect curves and to establish the most appropriate dose for research on radiation modifiers;
d) to examine the effects of the anti-inflammatory agent: L-alpha glycerylphosphorylcholine (GPC);
e) to define the Relative Biological Effectiveness of divers high LET radiation;
f) to characterize the effects of plateau and mid of the spread-out Bragg peak (mid-SOBP) proton radiation relative to that induced by clinical MV photon beam reference.

III. Material and methods

III.1. Animal model, embryo harvesting and maintenance

Adult fishes were maintained segregated by sex; with favorable conditions of the recirculation water system, fed three times a day on a varied diet, commercial dry fish food supplemented with freshly hatched brine shrimp (*Artemia nauplii*) according to standard procedures (Westerfield M, 2000).

Wild-type adult fishes (2 females and 3 males) were mated in embryo breeding tanks in the afternoon and the eggs were spawned the following morning. Viable embryos were washed with 0.1% methylene blue solution, sorted under a stereomicroscope (Stemi 508, Stand K LAB, Carl Zeiss), transferred to a 10 cm Petri dish, containing 5 ml E3 embryo medium and maintained under normoxic conditions at 27.5 °C.

The experimental protocol was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged (XXXII./1838.2015).
III.2. Method of establishment of the zebrafish embryo model for radiobiology

Experiments were performed on viable embryos, of 3-, 6-, and 24 hours post-fertilization (hpf). Embryos were sorted 1 embryo/well in standard 96-well polystyrene microplates in 250 µl medium. Embryos were exposed to γ-radiation of doses ranging between 0 Gy and 20 Gy, in 5 Gy increments. Irradiation was performed by a Teragam K-01 (SKODA UJP, Prague, Czech Republic) cobalt unit with an average energy 1.25 MV, source isocenter distance 80 cm. For achieving the required build-up effect, the plates were placed between two polymethyl methacrylate (PMMA) slabs of 2 cm thickness. The isocenter was positioned in the plates’ geometrical centers. In order to maximize the field homogeneity, half of the required doses was delivered by a 20 x 20 cm beam downward (gantry angle 0°), while the other half with an identical beam upward (gantry angle 180°). Irradiation time correction factors were used to compensate for the decay of the Cobalt-60 source. After radiation treatment, the embryos were kept at optimal conditions for a seven days observation period and were examined under a light microscope.

III.3. Studying the effects of the radiation modifier - GPC pre-treatment

GPC at 194 μM/L dose level was used to examine the radiation modifier effect on healthy tissues. Embryos were divided into four groups: control, GPC-treated, irradiated, GPC-treated followed by irradiation. They were incubated for 3 hours and exposed to 20 Gy at 24 hpf for survival and morphological analysis and to 10 Gy at 24 hpf for molecular examination. The irradiation was performed as described previously, in 96 well plates (1 embryo/well) with Cobalt-60 beam.

III.4. Method of RBE definition of different high LET sources

Fertilized embryos in the pharyngula period (24 hpf) were individually placed in wells of a 96-well plate, with 250 µl embryo medium, for conventional photon irradiation. For comparison of different radiation qualities found in different institutions, the embryos were used at the same stage of development were used, but regarding the neutron facilities, the embryos were floating in E3 medium in eppendorf tubes for the irradiation period.

6 MV photons, generated by a linear accelerator (Primus2 Siemens), was used as a reference beam, at 0 Gy, 5 Gy, 10 Gy, 15 Gy and 20 Gy dose levels.

Neutron irradiations were performed at the Budapest University of Technology and Economics, using the Training Reactor “pneumatic rabbit” system. All zebrafish embryo irradiation, occurred in the thermal channel at dose levels: sham irradiated, 1.25 Gy, 1.875 Gy, 2 Gy, and 2.5 Gy. The power of the reactor during the experiment was 200 W.
Cyclotron generated neutron exposures were implemented at Hungarian Academy of Sciences, Institute for Nuclear Research Atomki Institution. The zebrafish embryos were exposed to the mixed neutron-gamma field of the p(18 MeV)+Be fast neutron irradiation facility based on the MGC-20E cyclotron, at 0 Gy, 2 Gy, 4 Gy, 6.8 Gy, 8.12 Gy and 10.28 Gy dose levels.

III.5. **Method for studying the biological effects of the charged particle beam**

A fixed horizontal monoenergetic pencil-like proton beams in the energy range of 70 - 230 MeV was used at the experimental hall of the University Proton Therapy Dresden (UPTD). Embryos in 24 hpf developmental period, were irradiated with protons at the entrance plateau and in mid-SOBP. For 150 MeV protons a beam shaping system consisting of a double-scattering device and a ridge filter provides a laterally extended proton field of 10 x 10 cm² size and a SOBP of variable widths between 20 - 32 mm in water. Three independent experiment replications, were performed for each dose group irradiated with plateau and mid-SOBP protons, as well as clinical linac type Artiste (Siemens AG, Erlangen, Germany) at UPTD was used as a reference, the gantry was rotated for horizontal delivery of 6 MV photon beams comparable to the proton irradiation. Individual replications comprise 96 embryos per dose group of 0 Gy, 5 Gy, 10 Gy, 15 Gy, 20 Gy and 30 Gy.

III.6. **Measurements**

**Survival and morphology analysis**

In order to establish the age-related dose-response survival curves, to quantify the toxic and radiation modifier effects of GPC on survival, growth and morphological disorders, daily microscopic examination was performed at 24 hour intervals up to 7 days. The embryos were observed without any manipulation in the microplates and were incubated at 27.5°C with 14-h light/10-h dark cycle during the observation period. Viability was analyzed with light transmission using a Nikon Eclipse TS100 inverted microscope at 10X, 20X magnification and from the deformed specimens representative images were acquired at 10X magnification using a Nikon Coolpix 4500 (4.0 mega pixels 4X zoom) camera. Dead embryos were removed daily and the number of viable and malformed embryos were registered. Survival was calculated as a percentage of viable embryos to the total number of embryos exposed in each treatment group over time. The criterion of embryonic survival was the presence of cardiac contractions and blood circulation. Developmental status of the embryos, hatching rate, the size and shape of the embryos, skull, spine and tail, the development of the different organs eye, brain, yolk sac resorption, and pericardial edema were determined daily in the proportion of the living embryos.

Severe morphological disorders, like pericardial edema and spine curvature were evaluated as biological endpoints for the RBE definition.
Histopathology and tissue morphology evaluation

For histological assessments, the groups of control and treated embryos were sacrificed after 7 days of observation placed in 1:100 dilution of 4 mg/ml tricaine methanesulfonate then fixed by immersion (4% paraformaldehyde for 3 days), and embedded in paraffin. Transverse whole-body sections (4 μm thickness) were taken and stained with hematoxylin and eosin. The histology sections were scanned with Pannoramic MIDI digital slide scanner (3D-HISTECH Ltd., Budapest). Representative images were analyzed for evaluation and recorded using the Pannoramic Viewer 1.14.50 RTM 3DHISTECH at least 15 embryos, from each treatment group were observed with the program.

Quantitative Polymerase Chain Reaction (qPCR)

After 1 and 2 hours post-treatment, the inflammatory cytokine level was measured. To ensure adequate biological material for the measurement, three replicates were generated from each experimental group by pooling 20 embryos. Total RNA was isolated in Trizol using a homogenizer. Chloroform was then added to the homogenized embryo tissue. RNA was precipitated by adding isopropanol than the tubes were gently rotated, then incubated. Following centrifugation, the supernatants were collected. RNA concentration was measured by spectrophotometer. cDNA was synthetized and real-time PCR were performed.

IL-1β and NF-κB levels were measured using sequence-specific TaqMan gene expression assays with 18S rRNA used a pre-optimized primer and probe assay for endogenous control. Each PCR reaction was repeated and the relative mRNA level was calculated by the 2−ΔΔCT method.

Scoring system for quantitative analysis of embryo malformations

In order to categorize the pericardial edema and spinal curvature, a morphological scoring system was adapted (Brannen et al., 2010). Pictures recorded from the malformed embryos on the 3rd and 4th day post-irradiation (dpi) were retrospectively assessed and categorized in accordance to the scoring system. In the numerical scoring system for the pericardial edema, score of 1 represented the normal healthy state of the embryos, score 2 meant a variation within the normal range with a very small edema, score 3 a marked abnormality where the pericardial edema size was smaller than the head size and score 4 a major disorder with the size of the heart edema equal or even larger than the size of the head. For the spinal curvature score of 1 represented normal spine, score of 2 a curved end of tail, score of 3 a slight bending from half of the body, and the score of 4 the most severe curvature. The mean scoring values describing the average damage induced by one radiation quality were calculated on the basis of the scored malformations on the 3rd and 4th dpi.
III.7. Statistical analysis

For statistical evaluation of the data for the comparison of survival and the distortion curves of different radiation qualities, Log-rank test with Bonferroni correction, as well as Chi-square test were applied with GraphPad Prism Version 7.03 Windows.

Data assessment of the GPC radio-protection effects were performed using the Cox regression in R statistical programme language (R 3.2.2 for Windows). These data were expressed as mean ± standard deviation (SD). Levels of statistical significance were taken as p < 0.05.

Measurement of the level of inflammatory cytokine was performed in a commercial statistical software package SigmaStat. Non-parametric methods were used and the differences between groups were subjected to Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn’s method for pairwise multiple comparison.

For graphical representation of the average survival and the malformation rates of the proton irradiation, were determined as mean value of the three experiment replications, in dependence on dose the software Origin Lab 2017.

IV. RESULTS

IV.1. Age related radiation dose-response relationship

Daily assessment of embryo viability during seven days after irradiation showed a strong inverse correlation with the radiation dose. In case of 6 hpf irradiated embryos no relevant mortality occurred at 5 Gy dose level, whilst more than 80% was already dead at 96 hpf at 20 Gy. At day 7, half of the irradiated embryos were alive, therefore 15 Gy was established as the LD_{50} for embryos irradiated at 6 hpf. Embryos at 24 hpf were less susceptible to radiation, the LD_{50} was shown to be 20 Gy on day 7.

The number of incidence of serious embryonic developmental defects was proportional to the radiation dose and age of the embryos similarly to survival, major distortions occurred earlier if irradiation was performed at 6 hpf.

Histopathology

In the irradiated, observed individuals, the nervous tissue was normal but there were noticeable alterations in the ceratobranchials, in the skin, such as the disappearance of mucous cells and development of subcutaneous edema. There were decrease in goblet cell numbers. In relation to the hepatopancreatic interstitial edema, hydropic and simple pathologic signs of hepatocytes were seen in irradiated individuals. Pycnotic changes in the nuclei of the hepatocytes were also observed in the group irradiated with 20 Gy at 24 hpf. Dose dependent pericardial deterioration, such as slight hydropericardium were observed and this abnormality was more pronounced with increasing doses. Large amounts of mucous
and catarrhal were present in the intestinal flux in each irradiated group and cells with irregular shapes with a larger, hyperchromatic nuclei, as well as goblet cells were found in the intestinal mucous membrane in the all treated groups.

**IV.2. The effects of GPC on survival and distortion**

GPC in 194 µM/L concentration with 3 hours incubation prior to the radiation delivery had significant protective effects. On day 7, the difference in survival between the GPC pretreated and irradiated group and the irradiated control group was 20%, there were significant differences in survival as well as in the morphologic alterations compared with the control samples. Radiation-induced cell damage of the different organ systems revealed that the most severe deteriorations occurred at higher dose levels and there was an important radio-protective effect of GPC. The alterations, induced by ionizing radiation in the developing gastrointestinal system were reduced and partially restored by GPC pre-treatment.

**Histological evaluation**

In the groups irradiated with 20 Gy, large amounts of mucous, catarrhal were observed in the intestinal tract, as well as goblet cells were found in the intestinal mucous membrane. These were characterized by pseudo-multilayer epithelia, moderate disorganization of the columnar cells and the cytoplasm was wider in the intestinal lumen. These severe alterations, induced by ionizing radiation in the developing gastrointestinal system were reduced and partially restored by GPC pre-treatment.

**Inflammatory cytokine level measurement**

Whilst investigating the early phase of pro-inflammatory activation signal pathway it was found that the IL-1β expression was reduced to the control level in the pre-treated group and thus, the induction of the NF-κB pathway activation had been prevented. The results show that IL-1β and NF-κB are activated in response to injury at different times, when measured 1 hour and 2 hours after 10 Gy irradiation. This activation level was reduced significantly by GPC.

**IV.3. Survival for high LET-RBE definition**

The analysis of the survival curves resulted in LD$_{50/7}$ values of 2 Gy for fission neutrons, 8.12 Gy for cyclotron neutrons and 20 Gy for γ rays respectively. The ratio of the LD$_{n/50/7}$ for fission neutrons and LD$_{γ/50/7}$ photon beam provided an RBE of 10 and for LD$_{n/50/7}$ 18 MeV neutrons and LD$_{γ/50/7}$ reference photons of 2.5.

**RBE determination by malformation**

Developmental retardation and morphological changes (micro-ophthalmia, spine curvature, pericardial edema) of the living embryos were recorded with a simplified approach. Dose and LET dependent
morphological changes were observed. The distortion assessment on the 4\textsuperscript{th} and 5\textsuperscript{th} dpi provided evaluable data for different radiation quality comparison, which is in accordance with the survival based calculation.

\textit{Histopathology evaluation of different organs}

The histopathological assessment showed dose dependent tissue alteration in eye, brain, the gastrointestinal system, liver and muscles of the embryos after irradiation. The different LET ionizing radiation treatments caused considerable disorganization of the retinal layers, in contrast to the separable cellular layers. The radiation resulted in lens opacification, the loss of volume and there were observed a marked decrease in the diameter of the eye in embryos irradiated with fission neutron and cyclotron-based neutron sources, compared to the control. In case of control embryos brain, the neuropil is intact unlike in 20 Gy photon and 2 Gy groups using fission neutron and 8.12 Gy treated groups with cyclotron-based neutron sources where neuropil loosening were observed. In the diencephalon and in the medulla cell disorganization was found 7 days after treatment in the individuals exposed to 20 Gy photon and 8.12 Gy cyclotron-based neutron sources. In the groups irradiated with 20 Gy photon and 2 Gy fission neutron, the number of goblet cells were partially decreased and in case of cyclotron-based neutron at 8.12 Gy dose level they were almost completely depleted. The goblet cells particularly showed early signs of the effects of radiation and their number seemed to be well correlated to the delivered dose.

The tissue damages caused by high LET radiation were more pronounced at the same dose level corresponding to the LD\textsubscript{50} dose equivalent and it underlines the importance of an endpoint definition for RBE calculation.

\textit{IV.4. Proton treatment - RBE determination}

\textit{RBE by survival analysis}

On the first two days post-irradiation no significant impact was observed on embryonic survival for any doses lower than 15 Gy. Proton doses higher than 10 Gy for SOBP and 15 Gy for plateau irradiation significantly reduced survival levels at 4 dpi relative to the photon reference. This was also reflected in the LD\textsubscript{50}, which was about 30 Gy for SOBP protons and slightly higher for plateau protons. Concerning the embryonic survival, RBE values of 1.13 ± 0.08 and of 1.20 ± 0.04 were obtained at 4 dpi with 20 Gy of plateau and mid-SOBP protons relative to 6 MV photons.

\textit{RBE by malformation analysis}

The number of embryos with pericardial edema, as one of the acute reactions after irradiation, increased faster than the number of embryos with spine bending. At the 2\textsuperscript{nd} and 3\textsuperscript{rd} dpi for doses of 15 Gy and 20 Gy significant differences were predominantly found in the rates of pericardial edema between
proton and photon treatment. Doses higher than 15 Gy triggered pericardial edema in almost every treated embryo and a significant difference was found for the 15 Gy mid-SOBP irradiation group at the 4th dpi. For the induction of pericardial edema a dose distinction of 12.9 Gy for proton and 16.0 Gy for photon treatment was found at the 3rd dpi. For comparison, distinct dose levels of 18.6 - 22.1 Gy at the 3rd dpi and of 14.7 - 16.7 Gy at the 4th dpi were measured for spine deformations in 50% of the embryos after proton relative to photon treatment. On basis of the spinal curvature rates, observed at 4 dpi with 20 Gy protons RBE values of 1.25 ± 0.16 and of 1.10 ± 0.14 were calculated for the exposure in mid-SOBP and entrance plateau region relative to MV photons, respectively. Sigmoidal dose response curves became manifest for the pericardial edema as one example of acute radiation damage during the 4 day observation period. For spine curvature, a linear dose dependent increase of severity up to dose of 30 Gy was observed at 4th dpi probably caused by extensive cell death in the spine for doses above 10 Gy.

V. DISCUSSION

The increasing use of particle therapy and the emergence of innovative radiation methods raise the necessity of valid, reproducible preclinical data on the biological effects of these ionizing radiations. We established an in vivo, novel vertebrate model to examine the radiation-induced biological effects.

V.1. Age and dose-dependent survival curves

Few results have been published on fish embryo models (medaka, zebrafish) and they were used mainly for investigating on radiation protection aspects. In the course of the research, precise dose delivery techniques, adapted sample holders and the appropriate irradiation geometry of this promising model were determined. Thereafter the biological factors of the zebrafish embryos had been optimized for radiation biology research. To that aim examination of the effects of whole-body single fraction photon irradiation at different dose levels and different ages were performed. We exposed embryos in different post-fertilization time points (3 hpf, 6 hpf, 24 hpf) and evaluated several endpoints, such as survival, macro and micro morphologic alterations in the developing embryos. In our radiation model, the survival, the morphological deteriorations and the histological lesions proved to be age and dose-dependent. Younger embryos, especially before midblastula transition (MBT) i.e. at the age < 24 hpf were found to have not yet fully developed radiation damage repair proteins (McAleer et al., 2005), resulting in increased radiosensitivity, which was also confirmed by our experiments. Whereas the LD50 was 20 Gy for embryos irradiated with standard photon beam at 24 hpf, a dose of only 15 Gy caused embryonic mortality of half of the embryos on the 7th dpi in the group irradiated at 6 hpf. This strongly underlines that the zebrafish embryo model is highly suitable for radiation biology experiments. The 24 hpf embryos
proven to be highly stable, well reproducible and appropriate system for investigations on ionizing radiation effects and on potential radiation modifying agents.

V.2. Testing of potential radiation modifier

Phospholipids play a major role in rearrangement and reduction of toxicity against agents damaging the cell membrane but about the direct toxicity of administrated phospholipids at high concentrations little is known. GPC is a phospholipid derivative known to stabilize cell membrane function after inflicting damage, which statement proved to be true in our investigation. GPC reduced the rate, alleviated the number and severity of morphological abnormalities when administered before radiation treatment. Histopathological slides showed, that especially the radiation damage to the gastrointestinal tract was greatly reduced and even partially restored to normal with GPC pretreatment. As Westerfield (2000) described, major functional damage to the gastrointestinal system, like in the case of irradiation, will lead to death by starvation within 10 days after conception. GPC pre-treated embryos though, may be able to keep their normal nutritional capability. There is an evidence that the activation of canonical NF-κB pathway plays a significant role in inflammatory changes, induced by radiation in normal tissues in the developing vertebrate organism. The hypothesis of radioprotection by inhibiting inflammatory modulators like NF-κB was confirmed in the zebrafish embryo model, in which the NF-κB inhibitors ethyl-pyruvate and the synthetic triterpenoid CDDO-TFEA were given in combination with radiation (Daroczi et al., 2009). Therefore the NF-κB and IL-1β gene expression was studied, assuming that the observed protective effect of GPC may be due to the inhibition of this pathway. The essential pro-inflammatory cytokine of interleukin-1 family members (Vojtech et al., 2012) the IL-1β is rapidly induced after irradiation and this cytokine has also been implicated in edema shaping (Gaber et al., 2003). Overexpression of IL-1β is the first sign of inflammatory activation after IR, which induces the activation of the NF-κB pathway (Ogryzko et al., 2014). The addition of GPC reduced the IL-1β expression down to the control level and prevented the activation of the NF-κB pathway. Observation of modulation of early pro-inflammatory activation by GPC at a non-toxic concentration may provide an explanation for the radio-protective effect of the agent.

V.3. RBE determination of different high LET neutron sources

Being aware that in the earlier life stages embryos are more sensitive to the ionizing radiation, we investigated and compared the biological effectiveness of different radiation qualities using 24 hpf embryos. The RBE is known to be variable and influenced by different factors such as tissue type, biological endpoint, treatment regimen, ion type (Lühr et al., 2017). There has been a considerable amount of research performed in order to measure the RBE at different high LET sources at particle accelerator facilities worldwide using in vitro cell cultures, the gold standard of radiobiology (Beyreuther et al.,
In order to overcome the uncertainties of the \textit{in vitro} experiments, \textit{in vivo} systems that are clinically more relevant have been introduced in radiation research providing important data on the dose-dependent reactions of a complex organism. To that aim, embryos were exposed to reactor fission neutrons, cyclotron-based fast neutrons and to conventional photon reference beam (LINAC 6 MV photon). RBE definition of high LET beams relied on two quantitative endpoints (survival and malformation) and a detailed tissue damage, histological analysis resulted in similar results obtained using another fish species with different endpoint assessment. After comparing the survival curves we have observed that the biological effectiveness was 10 times higher for high LET thermal neutrons and 2.5 times higher for cyclotron-generated fast neutron beam. The result of the calculated RBE values was well supported by the experiments performed using broad energy spectra, mixed neutron-photon and monoenergetic neutron beams (Juerß \textit{et al.}, 2017). The high reliability of the zebrafish embryo survival assay was revealed during the repeated neutron irradiation experiments, therefore, survival analysis is considered as reliable tool for RBE measurements.

Microscopic studies on the embryo morphology \textit{in vivo}, and on histopathological slices proved the dose and LET dependent organ malformations (shortening of the body length, spine curvature, microcephaly, micro-opthalmia, pericardial edema and inhibition of yolk sac resorption) and marked cellular changes in eyes, brain, liver, muscle and in the gastrointestinal system.

All of the investigated tissues in the literature, the tail muscle, intestinal tract, central nerve system and eye exhibited clearly identifiable radiation related alterations, i.e. hypocellularity and disorganization of cellular layers in concordance with these findings.

\textbf{V.4. Biological effects of proton beam}

The increasing use of proton radiotherapy and the rising number of long-term survivors has given rise to a vital discussion on potential effects on normal tissues. The clinically applied generic RBE of 1.1 were only obtained by \textit{in vitro} studies, whereas indications from \textit{in vivo} trials and clinical studies are rare. The radiobiological effects along the charged particle path at two positions could be investigated with high spatial resolution due to embryos small size of about 0.5 – 1 mm. We therefore set out to characterize the effects of plateau and mid-SOBP proton radiation relative to that induced by clinical MV photon beam reference. The number of surviving embryos significantly declines at higher doses, for proton treatment more efficient than for photons, whereas for protons the delivery of 30 Gy resulted in a 50 \% survival rate (LD$_{50}$) already at the 4$^{th}$ dpi in our work. The dose threshold of 15 Gy for embryonic mortality observed at 4 dpi was also seen by others (McAleer \textit{et al.}, 2005), whereas for lower energy protons of 8 MeV higher
mortality rates were already revealed 4 days after treatment of 24 hpf wild type embryos with a dose of 6 Gy (Li et al., 2018).

Survival rates and the analysis of morphological abnormalities revealed that doses higher than 10 Gy were required to trigger the appearance of pericardial edema and spinal curvature within the follow up time of four days. Similar threshold doses of about 10 Gy were observed at 4 dpi in embryos treated during pharyngula stage with 60 Co γ-rays (Freeman et al., 2014). Pervasive and fast appearance of pericardial edema correlates with its emergence as acute inflammation reaction induced by cytokines released early after irradiation (Schaue et al., 2012). In contrast, deformations of the spine are probably caused by the apoptosis of neuronal cells in the developing spinal cord (Sayed and Mitani, 2016), which takes time to cause observable abnormalities after irradiation.

Based on embryonic survival data, RBE values of 1.13 ± 0.08 and of 1.20 ± 0.04 were determined four days after irradiations with 20 Gy plateau and SOBP protons relative to 6 MV photon beams. This has not been realized so far with aquatic animals. These values are in accordance with RBE values in the range of 0.96 – 1.13 and of 1.0 – 1.2 found in previous in vivo studies for treatments in the entrance plateau and mid-SOBP position (Uzawa et al., 2007), respectively. These RBE values were confirmed by relating the rates of embryos with morphological abnormalities for the respective radiation qualities and doses. In this context, a comparison of RBE obtained on basis of zebrafish embryonic survival rates to other in vivo results, which are most often based on measurements of acute or late effects of a single organ in rodents is critical (Saager et al., 2017, 2018).

In conclusion, our study validates the general applicability of zebrafish embryo as an alternative small vertebrate model for testing and assessing different radiation qualities, even under non-laboratory conditions. Qualitative endpoints such as survival and malformations can be used to describe the overall effect of radiation on the whole organism. The potential application of zebrafish embryos for spatially resolved RBE measurements along the proton depth dose distribution seems to be conceivable.
VI. CONCLUSION AND FINDINGS

a) We have founded the first Zebrafish laboratory in Szeged and we have contributed significantly to the establishment of a novel vertebrate model for radiobiology research. The fish embryo system proved to be appropriate for investigating the effects of different ionizing radiation on a large scale and for preclinical studies on potential radio-protective agents.

b) Exact and controlled dose delivery techniques and adapted radiation setup of zebrafish embryos for special technical conditions including limitations had been worked out.

c) The proper embryonal age, observation time points for assessment of the different biologic endpoints, such as survival, reliable quantitative morphological analysis, and complex evaluation of histopathologic and molecular changes could be defined. We derived well-reproducible dose-response curves and LD$_{50}$ for each radiation quality.

d) In our experiments GPC exhibited protective effects against radiation induced lethality, multi-organ morphological and histological impairment. Our results suggest that the inhibition of early radiation induced activity of pro-inflammatory pathway activation could be a potential mode of action of GPC. GPC may therefore be a possible future candidate for protection of the normal tissues exposed to incident IR during cancer radiotherapy.

e) Viability and malformation detection has been shown to be a responsive measure for all radiation types. We have defined the RBE for fission and cyclotron based neutron as well as for proton sources.

f) The potential application of zebrafish embryos for spatially resolved RBE measurements along the proton depth dose distribution proved to be conceivable.

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