Prilocaine Hydrochloride Protects Zebrafish from Lethal Effects of Ionizing Radiation: Role of Hematopoietic Cell Expansion

Manali DIMRI[*1], Jayadev JOSHI[*1], Nitisha SHRIVASTAVA[*1], Subhajit GHOSH[*1], Rina CHAKRABORTI[*2] and Prem Kumar INDRACANTI[*1]

[*1]*Radiation Biosciences Division, Institute of Nuclear Medicine and Allied Sciences*
[*2]*Department of Zoology, Delhi University*

(Received September 2, 2014; Accepted December 16, 2014)

Drug repositioning is an approach of significant translatability, and the present study was undertaken to screen a collection of FDA approved small-molecule clinical compounds for identification of novel radioprotective agents. Screening of JHCCL (Johns Hopkins Clinical Compound Library), a collection of 1,400 FDA approved small molecules, lead to identification of prilocaine hydrochloride, a local anesthetic used widely during dental procedures, as a potential radioprotector. Prilocaine, at a concentration of 20 µM, protected zebrafish from radiation induced (20 Gy) pericardial edema (PE), microphthalmia and rendered 60 % survival advantage over radiation exposed controls. While 40 % survival advantage over radiation exposed controls was achieved with 10 µM prilocaine. Prilocaine, in a dose-dependent manner, scavenged, radiation-induced hydroxyl radicals and maximally (43 %) at the highest concentration (1 mM) tried in this study. However, prilocaine exerted a mild superoxide anion scavenging potential (around 5 %) at all the concentrations used within this study. Prilocaine, at 20 µM concentration, significantly increased erythropoiesis, a marker for HSC function, in caudal hematopoietic tissue (CHT) in wild type and anemic zebrafish embryos (1.48 and 0.85 folds respectively) when compared to untreated (1) and phenylhydrazine (PHZ) (0.41 fold) treated control groups respectively. These results suggest that prilocaine is a radioprotective agent and free radical scavenging and HSC expanding potential seems to be contributing towards its radioprotective action.

Key words: Drug repurposing, zebrafish, beta blocker, hematopoietic stem cells, radioprotection

INTRODUCTION

Ionizing radiations like gamma and X-rays cause damage to the biological system through generation of free radicals and oxidative stress. The damage ranges from mortality to long-term effects in the form of induction or enhanced sensitivity to a variety of chronic diseases [1–3]. At the molecular level, oxidative damage to DNA, protein and lipids leads to downstream stress related responses, including cell cycle arrest, apoptosis [4]. In view of health concerns associated with radiation exposure scenarios like terrorist attacks, reactor accidents and ever expanding medical application of radionuclide's, development of clinically acceptable radiation countermeasure agents holds strategic importance [2]. Earlier studies have established that molecules of both synthetic and natural origin in isolation or as a combination have been reported to exert protection against lethal doses of ionizing radiation in a variety of model organisms [5]. A spectrum of different mechanisms has been attributed for radioprotection, and it includes free radical scavenging, antioxidant, metabolic modification, DNA repair, HSC proliferation [6]. However, a clinically acceptable agent for protection against lethal doses of ionizing radiation is still eluding. Another key hurdle in their clinical utility is that any novel molecule identified from screen needs to undergo the set of clinical trials prior to applications and due to the towering attrition in phase I clinical stages, routine drug discovery programs suffer money and time loss [7]. In view of this, drug repositioning is an approach of high translatability and success stories, including novel applications for thalidomide, fluoxetine, etc. have renewed interest in identifying leads for other important human diseases [7]. Screening of small-molecule clinical compound library for identification of novel molecules with radioprotective action is an approach of promising translatability [7, 8]. Target based screening of large collection of compounds using cell lines (in vitro) as model system has yielded promising molecules with potential therapeutic implications. Nevertheless, cell lines fails to mimic the physiological context and more importantly in neuro-endocrine context, which significantly dictates success in pre-clinical and clinical stages. Mice are a valuable model system for the understanding patho-physiology of human diseases. However, the size, labor, time requirements make it cost prohibitive for conducting small-molecule screens. Hence, there is a compelling need for identifying novel animal models, which satisfy the in vivo context and amenability for high or medium throughput screening. Zebrafish, tiny tropical teleost, has made a significant impact in biomedical research, and it has been put to use to study the aspects of biomedical significance [9].

Prem Kumar INDRACANTI, Radiation Biosciences Division, Institute of Nuclear Medicine and Allied Sciences (INMAS), Defense Research and Development Organization (DRDO), Brig SK Majumdar Road, Timarpur Delhi-110054, India Tel: 91-11-23905301 Fax: 91-9958456999 Email: prem_indra@yahoo.co.in

High fecundity, lesser husbandry cost along with advantages like rapid development, availability of genetic resources, ease for the development of transgenic lines and amenability for medium throughput screening has made zebrafish a valuable model organism for both basic and applied biomedical research [10]. Comparison of zebrafish and human reference genome revealed that 70 % of human genes have at least one obvious zebrafish orthologue [11]. Developing and adult zebrafish have also been used for screening and identification of radioprotectors [12]. Currently a number of small-molecule compound libraries are available commercially and in the present study, JHCCL was used for screening and identification of potential radioprotectors using developing zebrafish as a model organism. JHCCL is a collection of FDA approved small-molecule drugs and of all the existing drug libraries, this library contains 1400 FDA-approved drugs (41% of approved compounds; 1400 out of 3400) (http://htc.wustl.edu/library/JHCCL.html, http://remarag.org/drug_screen.html) [13]. JHCCL also include small-molecule clinical compounds, which have not yet been screened for the aforesaid purpose, making it a unique collection for studying radiation modulation.

MATERIAL AND METHODS

Small molecule library

JHCCL (ver1. 2) was obtained from Johns Hopkins University, USA and all chemicals, otherwise mentioned, were obtained from Sigma, MO, USA.

Zebrafish husbandry, embryo collection

Wild type (AB) zebrafish embryos were maintained at INMAS zebrafish facility on a 14 hour light/10 hours dark cycle at 28.5°C and all the experiments were carried out in accordance to the animal ethics guidelines of INMAS, Delhi, India (8GO/a/99/CPCSEA). Embryos were collected, staged and maintained in embryo medium (E3 medium; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) at 28.5°C. For better visualization of internal structures in some experiments, embryos were incubated with 0.003 % 1-phenyl-2-thiourea (PTU) to inhibit pigment formation.

Radiation exposure and small-molecule treatment

Wild-type age matched zebrafish embryos (20 hours post fertilization (hpf)) were arrayed in 48 well plate (n = 10) and small molecules from JHCCL were added at a concentration of 10 µM, 30 minutes prior to exposing to 20 Gy gamma radiation (60 Co source; Gamma cell 5000, AERB, India; dose rate 1.02 kGy/h). Post irradiation, embryos were incubated at 28.5°C for 24 hours, dechorionated manually and maintained till 96 hpf to evaluate the morphology and survival. Photomicrography of representative embryos was performed using a Olympus stereo zoom microscope (SZX16, Olympus, USA) at 100X magnification. Further, dose optimization was done for prilocaine for radioprotection.

Quantification of pericardial edema and microphthalmia

Embryos positioned laterally were surveyed for eye size and the presence of PE and imaged using a Olympus microscope (SZX16, Olympus, USA) at 100X magnification and morphometric analysis of images was performed using Image J software, and the area of the eye and PE was measured in pixels and used in the further analysis.

Hydroxyl radical scavenging assay

Different concentrations of prilocaine or mannitol was added to phosphate buffer saline (PBS) containing 2.8 mM of 2-deoxyribose (DR) in a final volume of 1 ml, and the mixture was exposed to 200 Gy gamma radiation. The extent of 2-DR degradation was measured by following TBA method [14]. To the mixture, 1 ml of thiobarbituric acid (TBA) (1%, w/v in 0.05 M NaOH) and 1 ml of 2.8 % trichloro acetic acid (TCA) was added and heated in boiling-water bath for 15 minutes. Thereafter, the samples were cooled and the absorbance of the chromogen formed was measured at 532 nm using a microplate reader (Spectramax M2e, Molecular Devices, USA) and results were calculated as % inhibition. Mannitol was used as a standard free radical scavenger.

$$\% \text{ Inhibition} = [(\text{Control OD} - \text{Test OD})/\text{Control OD}] \times 100$$

Superoxide anion scavenging activity

For assessing superoxide anion scavenging potential, different concentrations of prilocaine was added to 1 ml of nitroblue tetrazolium solution (156 µM NBT in 100 mM phosphate buffer, pH-7.4; 1 ml NADH solution (468 µM in 100 mM phosphate buffer, pH-7.4), and mixed thoroughly. Thereafter, 100 ml of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH-7.4) was added to initiate the reaction and the reaction mixture was incubated at 25°C for 5 minutes, and the absorbance at 560 nm was measured against the blank. % inhibition of OD was calculated by using the formula mentioned earlier [15].

Chemically induced hemolytic anemia and quantification of erythrocytes

Hemolytic anemia was induced by incubating the dechorionated and staged embryos (33 hpf) in E3 medium containing 0.5 µg/ml of PHZ until 48 hpf. Thereafter, the embryos were washed thoroughly using E3 medium and, at 54 hpf, arrayed (n = 10 per treatment group) in 48 well plate and exposed to prilocaine (20 µM) and incubation were further continued until 96 hpf. O-dianisidine (ODA) staining was used for quantifying the erythrocytes in CHT [16]. Briefly, at 96 hpf the embryos were washed and incubated in ODA staining buffer containing 0.6 mg/ml of o-dianisidine hydrochloride in 10 mM sodium acetate (pH-5.2) and 40 % ethanol. The staining reaction was activated by adding hydrogen peroxide (20 µl/ml; 30 % stock) and incubated at room temperature in the dark for 15 minutes, followed by extensive washing in PBS and fixing in 4 % paraformaldehyde (PFA). Embryos were positioned laterally, and images were captured (200X) using a bright field microscope (Dewinter, Italy). Quantification of erythrocytes was

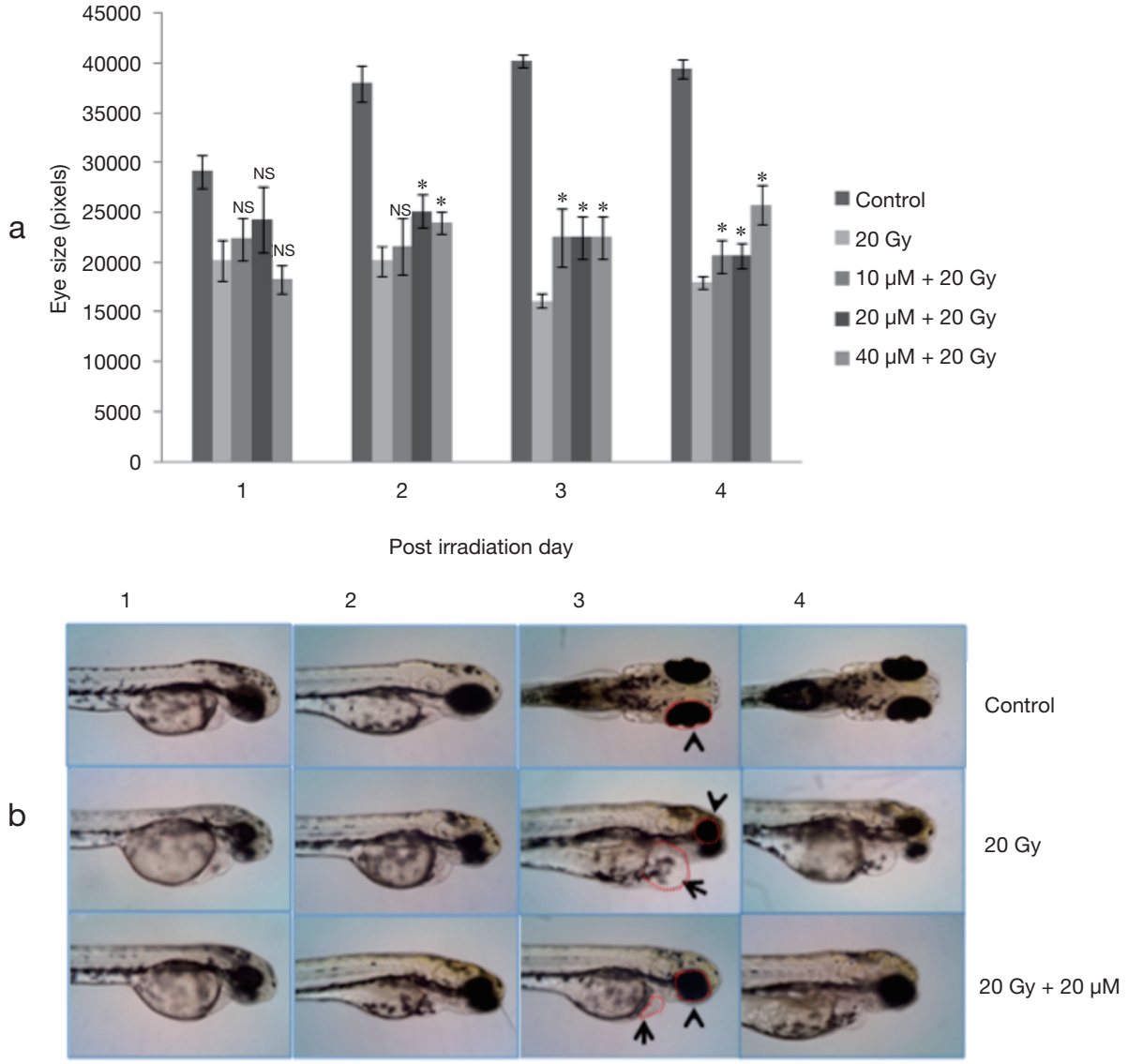

Fig. 1 a. Effect of varied concentrations of prilocaine on radiation (20 Gy) induced effects on eye size. Embryos exposed to 20 Gy gamma radiation in the presence or absence of prilocaine and changes in the eye size was observed till 4th post irradiation day. b. Representative images of different treatment groups are shown in different post irradiation days. The eye (arrow head) and pericardial edema (arrow) evident within different treatment groups is highlighted with the dotted line. Asterisk (*) indicates the p value < 0.05, when compared to radiation alone. NS not significant when compared to radiation alone group.

done by converting grayscale images to binary images and erythron pixels were quantified as integral density using Image J software (www.imagej.¬nih.¬gov/¬ij/, NIH, Bethesda,USA).

STATISTICAL ANALYSIS

Data is presented as Mean ± SD of a minimum of three independent experiments and significance was assessed by student's t-test and a p-value less than 0.05 was considered significant.

RESULTS

Screening of library

Screening of JHCCL at a concentration of 10 µM using zebrafish survival as an endpoint yielded a number of small molecules effective in rendering protection against 20 Gy gamma radiation. Among the effective hits, prilocaine was further investigated in detail.

Modulation of radiation-induced damage and lethality by prilocaine

Wild-type embryos without any treatment exhibited a continuous increase in the eye size (100 % embryos exhibited this phenotype) until 3 dpf (days post fertilization) and thereafter, no significant increase in eye size observed during the entire experimental duration (Figs. 1a & b). The eye size reduction and PE used in the present study as end points, to evaluate the radioprotection efficacy of the molecules, have been associated with poor development of the embryos, and are the broad-spectrum responses to toxicants.

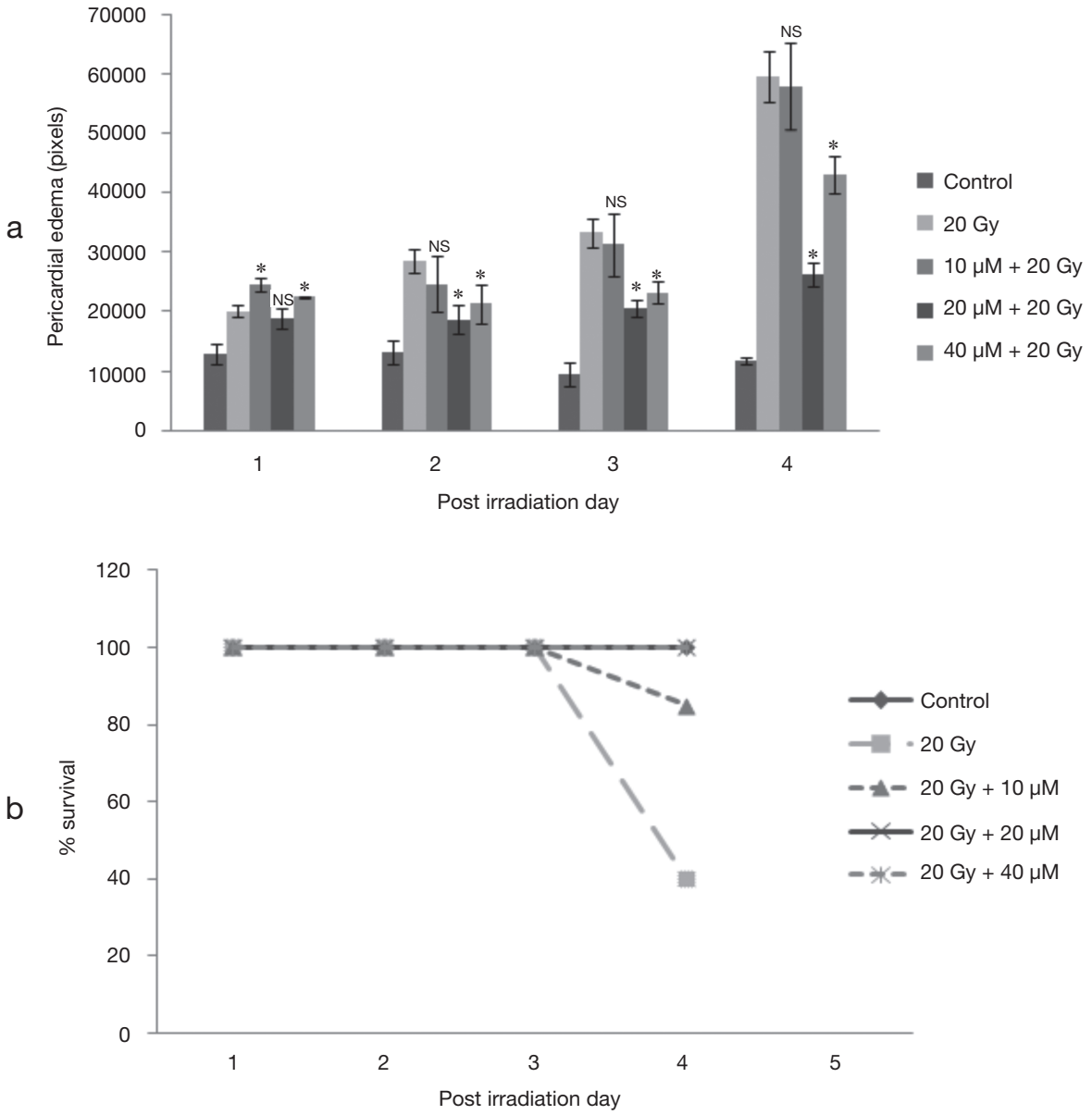

Fig. 2 Effect of varied concentrations of prilocaine on radiation (20 Gy) induced pericardial edema. a. Embryos were exposed to 20 Gy gamma radiation in the presence or absence of prilocaine, and the formation of pericardial edema was observed till 4th post irradiation day. b. Effect of prilocaine on radiation-induced mortality in zebrafish embryos. 20 hpf embryos were exposed to 20 Gy gamma radiation in the presence or absence of prilocaine, and survival was monitored for till the 4th post irradiation day. Asterisk (*) indicates the p value < 0.05, when compared to radiation alone. NS not significant when compared to radiation alone group.

Radiation exposure of 20 hpf staged embryos to 20 Gy gamma radiation lead to a significant slowing down of eye development and eye size (> 85 % embryos) in comparison to untreated controls (Figs. 1a & b). The size reduction was evident during the entire course of the experiment. Prilocaine (10, 20, or 40 µM) when added 30 minutes prior to irradiation, prevented the radiation-induced reduction of eye size and the protective effect (> 80 % embryos) was clearly pronounced from 3 dpf until the end of the experiment. Among different concentrations tried, prilocaine exerted a maximal protective effect at 40 µM. Similarly, 20 Gy gamma radiation exposure led to a significant increase in the area of pericardial edema (> 85 % embryos) in comparison to untreated control groups and exhibited a continuous increase throughout the experiment (Figs. 2a & 1b). There was a steep increase to the edema size on day 4 after irradiation. Pre-irradiation administration of prilocaine (10, 20, or 40 µM) reduced radiation-induced edema formation, and the protective effects were evident until the end of the experiment (> 85 % embryos). Prilocaine optimally protected zebrafish from radiation-induced PE at a concentration of 20 µM. Embryos without any treatment exhibited normal development without any gross deformities while embryos exposed to 20 Gy gamma radiation resulted in severe deformities, including reduction in eye size, PE, microcephaly, cup shaped spine, yellow pigmentation and stunted growth (> 90 % embryos). These deformities were found to increase in severity with time. In

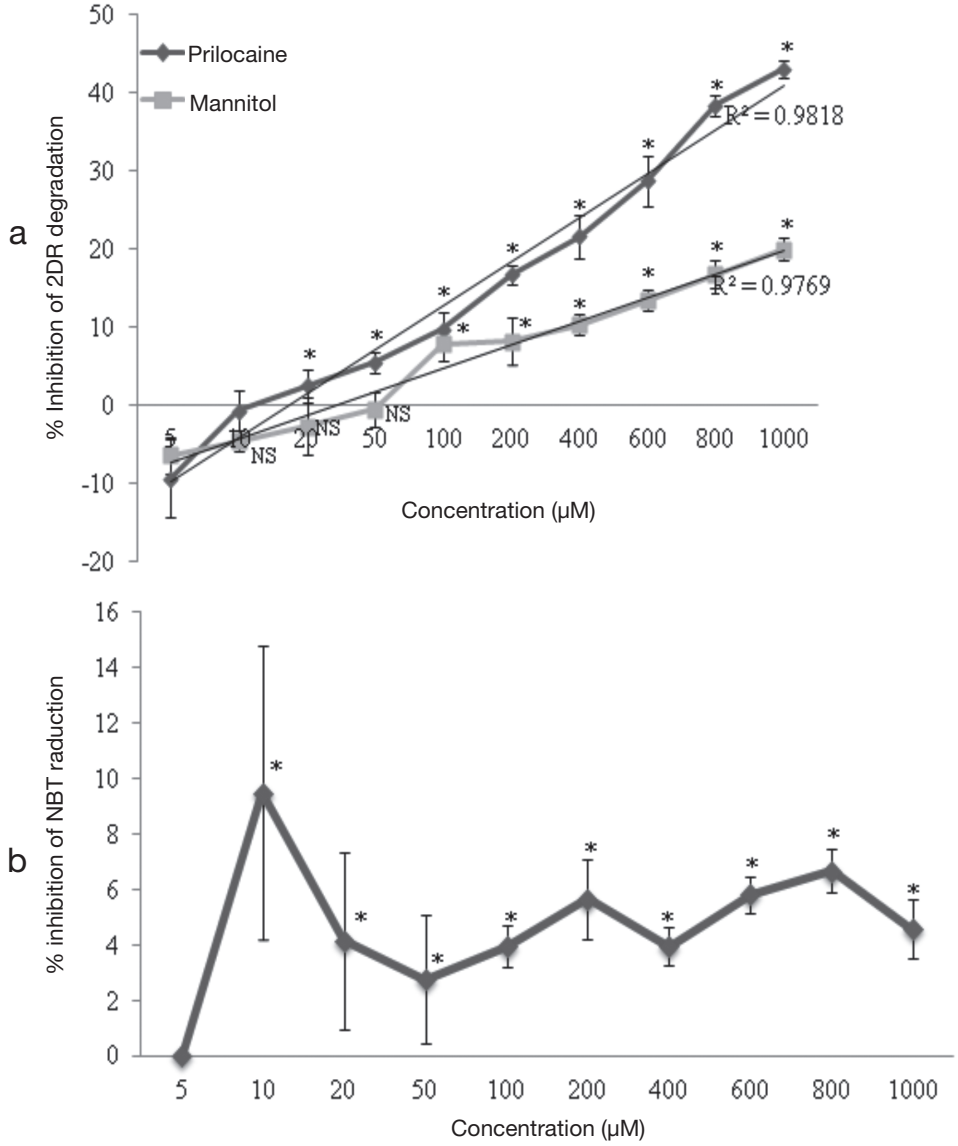

Fig. 3 a. Radiation induced OH radical scavenging potential of prilocaine or mannitol at varying concentrations. % Inhibition of chromogen was calculated with respect to control, which was considered as zero inhibition. Asterisk (*) indicates the p value < 0.05, when compared with radiation control. b. The effect of prilocaine on chemically generated superoxide anions, measured as % inhibition of NBT reduction and chromogen formation. Asterisk (*) indicates the p value < 0.05, when compared with a total NBT reduction.

the present study, in case of 20 Gy gamma radiation exposed embryos, 60 % of embryos were found to be dead by 4th post irradiation day (Fig. 2b). Prilocaine (20 or 40 µM) when administered 30 minutes prior to irradiation protected embryos from lethality and rendered > 80 % survival advantage (Fig. 2b). Apart from the survival advantages, the embryos showed overall less severe deformities (> 85 % embryos) (Fig. 1b).

Hydroxyl radical and superoxide anion scavenging by prilocaine

Exposure of 2-DR solution to 200 Gy gamma radiation resulted in its degradation and formation of thiobarbituric acid reactive substances, which in the presence of TCA interacted with TBA and formed pink chromogen. Prilocaine, in a dose-dependent fashion, inhibited hydroxyl radical mediated 2-DR degradation and chromogen formation (Fig. 3a). Maximum inhibition (43 %) was observed at the highest concentration tried in this study (1000 µM). Mannitol, standard free radical scavenger, did not exhibit hydroxyl radical scavenging at lower concentrations (below 50 µM) (Fig. 3a). However, mannitol significantly scavenged and reduced radiation-induced 2-DR degradation at concentrations more than 50 µM, but the radical scavenging efficacy was found to be less effective than prilocaine. However, prilocaine at all the concentrations tried in this study exhibited a mild superoxide anion scavenging action (5 %; Fig. 3b).

Effect of prilocaine on chemically induced hemolytic anemia and erythropoiesis in wild-type zebrafish embryos

PTU (0.003 %) that was used to block the pigmentation in developing embryos did not affect the definitive hematopoiesis (Figs. 4a & b). Zebrafish embryos ex-

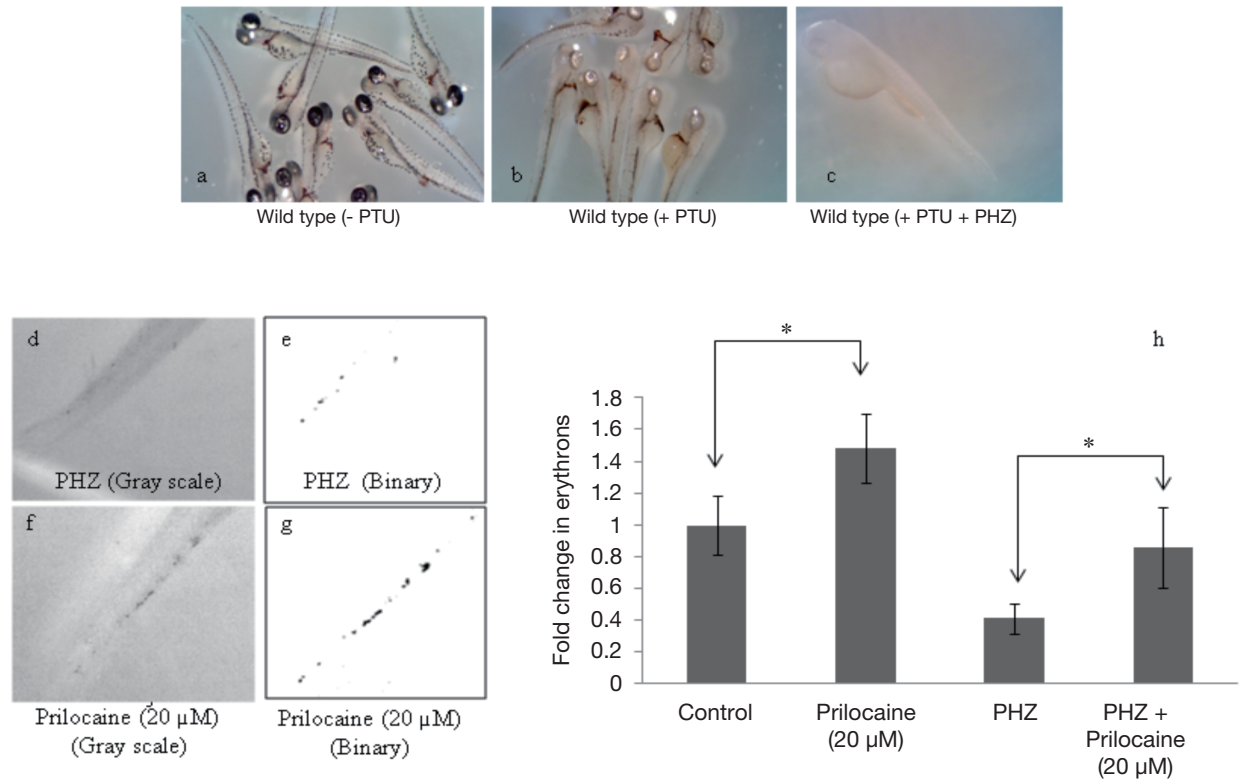

Fig. 4 Effect of prilocaine on recovery from chemically induced hemolytic anemia in wild-type zebrafish embryos. a. Left panel; Representative images of o-dianisidine stained wild-type embryos without (a) or with (b) PTU. Representative image of PHZ treated wild-type embryos is shown in c. d-g. The approach used for quantification of erythrons. Grayscale images were converted into binary images and quantified using Image J software. h. PHZ and control embryos were treated with prilocaine (20 µM) and monitored for the formation of mature erythrocytes in caudal hematopoietic tissue (CHT) at 96 hpf by staining with o-dianisidine Asterisk (*) indicates p value < 0.05, when comparison was made between respective groups. NS, not significant.

posed to 0.5 µg/ml of PHZ from 33 to 48 hpf showed complete loss of circulating erythrocytes and anemia by 72 hpf (Fig. 4c). Removal of PHZ led to recovery of erythropoiesis by 120 hpf (data not shown). At 96 hpf, PHZ treated embryos showed few mature erythrocytes in the CHT (0.34 folds in comparison to the untreated control group; Figs. 4d–h). Prilocaine at a concentration of 20 µM significantly enhanced erythropoiesis at CHT region (1.48 folds) in comparison to untreated wild-type embryos. Prilocaine also increased erythropoiesis in the CHT region (0.85 folds) in comparison to anemic control (0.34 folds).

DISCUSSION

The mid blastula transition stage of developing zebrafish has been reported to be the most radiosensitive stage and suitable for screening agents for radiation protection [17]. However, in this study 20 hpf embryos were used for radioprotector screening as a significant number of embryos either die or do not develop normally during 24 hpf and those which develop normally by 24 hpf attain adulthood. In the present study, 96 hpf were considered as the time limit for assessing both the mortality and organ damage as initial six days the embryos are independent of external feeding which minimize the variability in terms of differences in feeding ability post irradiation. In fact, similar screening schedule in zebrafish was used by several investigators for identification of radioprotectors [17–19]. The screening identified a number of small molecules with radioprotective potential, and a number of molecules were found to be either sensitize or had no significant effect on induction or recovery of radiation-induced damage (data not shown). Among the molecules which rendered radioprotection, prilocaine, a local anesthetic of the amino amide type first, was found to be novel with significant radiomodulatory effect. However, it does not rule out the possibility of other molecules being more potential, as the screening concentration (10 µM) tried may not be optimal for these compounds. Further studies are warranted to identify the optimal concentrations for these compounds. Prilocaine, at a concentration of 20 µM, significantly protected the embryos, both from lethality and radiation-induced organ toxicity, assessed as pericardial edema and eye size (Figs. 1 & 2) indicating its potential to act as a radioprotector.

To decipher possible mechanisms contributing to its radioprotective action, ability to scavenge radiation-induced free radicals was assessed. Radiation mediated damage involve the generation of free radicals, oxidative damage to macromolecules and cell death and free radical scavenging has emerged as one of the potential radioprotective mechanisms [1, 5]. The 2-DR assay is a reliable cell free system, widely in use, for assessing hydroxyl radical scavenging potential of an agent [14]. Prilocaine inhibited hydroxyl radical mediated degradation of 2-DR, suggesting its radical scavenging

potential (Fig. 3a). In fact these results in corroboration with earlier reports showing radical scavenging potential of prilocaine [20]. Prilocaine, at radioprotective concentration (20 µM), significantly scavenged hydroxyl radicals, strongly suggesting its possible contribution towards its radioprotective action (Fig. 3a). However, prilocaine exerted mild superoxide anion scavenging potential, another free radical generated by ionizing radiation. Free radical scavengers and antioxidants are well known to exert radioprotection when given prior to irradiation. However, in case of post irradiation scenarios mechanism like DNA repair, anti-inflammation, hematopoietic stem cell proliferation, etc. are critical for recovery of the exposed organism. The pioneering work by Lorenz et al (1951) has clearly established that transplantation or shielding of blood forming tissues was sufficient for achieving protection against lethal doses of ionizing radiation [21]. Now it has been well established that transplantation of the rare HSCs is sufficient for survival against radiation. Stem cells are characterized by their ability to self renew and differentiate into progressively restricted cells with specific cell fate. It is well understood that agents, which can expand HSCs have tremendous applications in the management of a number of life-threatening diseases, including radiation over exposure scenarios [21, 22]. In view of the promising therapeutic advantages HSC holds, efforts are on to identify agents which can expand HSPCs both in vivo and ex vivo [20, 23]. A number of agents of both synthetic and natural origin have been found to expand HSC ex vivo and in vivo, and some of the important ones include prostaglandin E2 [24], stem reginin1 [25], garcinol [26]. In view of this, it was considered interesting to study the ability of prilocaine to expand hematopoietic cell compartment. PHZ has been well established to induce reversible hemolytic anemia in a variety of species [27]. Free radical generation, oxidative stress and apoptosis in erythrocytes are some of the events in the hemolytic anemia cascade [27]. In case of hemolytic anemia, loss of mature erythrocytes leads to feedback signaling, which leads to increased formation of erythrocytes [28]. PHZ induced hemolytic anemia is a valuable approach for assessing the HSPCs expanding potential of different agents [28, 29]. PHZ induced hemolytic anemia is reversible in nature and the anemic state persists as long as the chemical is in the medium. Removal of PHZ leads to a recovery phase and normal levels of erythrocytes are achieved by a period of 72 hours in zebrafish embryos [29]. Preliminary studies carried in our laboratory suggested 96 hpf as the earliest time point where the differences between the normal and drug induced recovery could reliably be differentiated on the basis of o-dianisidine staining (20). Hence, 96 hpf was chosen as the time point for drug induced recovery studies and prilocaine, at a concentration of 20 µM, significantly accelerated erythropoiesis (Fig. 4) suggesting its ability to modulate HSC compartment. Overall, the results suggest that prilocaine has antioxidant and HSC expanding potential which might be contributing towards its radioprotective action. Since, in the present study prilocaine was added prior to irradiation, the radioprotective manifestation could be majorly attributed towards its free-radical scavenging and antioxidant action with HSC expanding potential as a contributory mechanism responsible for post irradiation recovery. Further, mitigative studies with prilocaine are warranted to assess the contribution of HSC expanding potential towards its radioprotective action. The present study suggests radioprotective action of an approved local anesthetic drug.

Recently lidocaine, another local anesthetic drug, has been reported to render protection against radiation-induced damage to salivary glands [30]. As prilocaine is structurally closer to lidocaine it is interesting to evaluate its protective action towards radiation-induced salivary dysfunction. However, validation of HSC expansion and radioprotective potential in murine and higher mammals, including non-human primates holds key for translating prilocaine for possible utility in the management of radiation over exposed victims.

AUTHOR DISCLOSURE STATEMENT

The authors declare no competing financial interests.

REFERENCES

1) PremKumar I, Gupta D, Bhatt AN, Dwarakanath BS. Models for the Development of Radiation Countermeasures. Def Sci J 2011; 61: 1-8.
2) Dörr H, Meineke V.Acute radiation syndrome caused by accidental radiation exposure - therapeutic principles. BMC Med 2011; 9: 126.
3) Xiao M, Whitna MH. Pharmacological countermeasures for the acute radiation syndrome. Mol. Pharmacol 2009; 2: 122-133.
4) Pogozelski WK, Xapsos MA, Blakely WF. Quantitative assessment of the contribution of clustered damage to DNA double-strand breaks induced by 60 Co gamma rays and fission neutrons. Radiat Res 1999; 151: 442-448.
5) Greenberger JS, Clump D, Kagan V, Bayir H, Lazo JS, Wipf P, Li S, Gao X, Epperly MW. Strategies for discovery of small molecule radiation protectors and radiation mitigators. Front in Oncol 2012; (1): 59.
6) Weiss JF, Landauer MR. Radioprotection by antioxidants. Ann N Y AcadSci 2000; 899: 44-60.
7) Ashburn TT, Thor KB. Drug repositioning: Identifying and developing new uses for existing drugs. Nature Rev Drug Discov 2004; 3: 673-683.
8) Kim K, Pollard JM, Norris AJ, McDonald JT, Sun Y, Micewicz E, Pettijohn K, Damoiseaux R, Iwamoto KS, Sayre JW, Price BD, Gatti RA, Mcbride WH. High throughput screening identifies two classes of antibiotics as radioprotectors: Tetracyclins and fluoroquinolones. Clin Cancer Res 2009; 15: 7238-7245.
9) Ablain J, Zon LI. Of fish and men: using zebrafish to fight human diseases. Trends Cell Biol 2013; 23(12): 584-586.
10) Das BC, McCormick L, Thapa P, Karki R, Evans T. Use of zebrafish in chemical biology and drug discovery. Future Med Chem 2013; 5(17): 2103-2116.
11) Howe K, Clark MD, Carlos F. Torroja CF *et al*. The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013; 496: 498-503.
12) Epperly MW, Bahary N, Quader M, Dewald V, Greenberger JS. The zebrafish-Danio rerio is a useful model for measuring the effects of small-molecule mitigators of late effects of ionizing irradiation. In Vivo 2012; 26(6): 889-897.
13) Chong CR, Chen X, Shi L, liu JO, Sullivan DJ. A clinical drug library screen identifies astemizole as an antimalarial agent. Nat Chem Biol 2006; 2(8): 415-416.
14) Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radical. Anal Biochem 1987; 165: 215-219.
15) Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide

anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun. 1972; 46(2): 849–54.
16) Ransom DG, Haffter P, Odenthal J, Brownlie A, Vogelsang E, Kelsh RN, Brand M, van Eeden FJM, Furutani-Seiki M, Granato M. *et al*. Characterization of zebrafish mutants with defects in embryonic hematopoiesis. Development 1996; 123: 311–319.
17) Geiger GA, Parker SE, Beothy AP, Tucker JA, Mullins MC, Kao GD. Zebrafish as a "Biosensor"? Effects of Ionizing Radiation and Amifostine on Embryonic Viability and Development. Cancer Res 2006; 66(16): 8172–8181.
18) Daroczi B, Kari G, McAleer MF, Wolf J C, Rodeck U, Dicker AP. In vivo Radioprotection by the Fullerene Nanoparticle DF-1as Assessed in a Zebrafish Model. Clin Cancer Res 2006; 12(23): 7086–7091.
19) Dimri M, Joshi J, Chakraborti R, Sehgal N, Angara S, Indracanti P. Todralazine protects zebrafish from lethal effects of ionizing radiation: role of hematopoietic cell expansion. Zebrafish. 2014 (Accepted).
20) Günaydin B, Demiryürek AT. Effects of prilocaine and articaine on human leucocytes and reactive oxygen species in vitro.Acta Anaesth Scand 2001; 45: 741–745.
21) Lorenz E, Uphoff D, Reid TR, Shelton E. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J Nat Cancer Inst 1951; 12: 197–201.
22) Ding S, Schultz PG. A role of chemistry in stem cell biology. Nat Biotech 2004; 22(7): 833–840.
23) Grosselin J, Sii-Felice K, Leboulch P and Tronik-Le Roux D. Searching for the Key to Expand Hematopoietic Stem Cells, Advances in Hematopoietic Stem Cell Research, Dr. Rosana Pelayo (Ed.), ISBN: 978-953-307-930-1, In Tech, Available from: http://www.intechopen.com/books/advances-inhematopoietic-stem-cell research/searching-for-the-key-to-expand-hematopoietic-stem-cells.
24) North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR *et al*. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature 2007; 447(7147): 1007–1011.
25) Boitano AE, Jian Wang J *ET AL*. Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells. Science 2010; 39: 1345–1348.
26) Nishino T, Wang C, Mochizuki-Kashio M, Osawa M, Nakauchi H, Iwama A. Ex Vivo Expansion of Human Hematopoietic Stem Cells by Garcinol, a Potent Inhibitor of Histone Acetyl transferase. Plos One 2011; 6(9): e24298.
27) Berger J. Phenylhydrazine haematotoxicity. J Appl Biomed 2007; 5: 125–130.
28) Takizawa H, Schanz U, Manz MG. Ex vivo expansion of hematopoietic stem cells: mission accomplished? Swiss Med Wkly 2011; 141: w13316.
29) Ferri-Lagneau KF, Moshal KS, Grimes M, Zahora B, Lishuang L, Sang S, Leung TC. Ginger Stimulates Hematopoiesis via Bmp Pathway in Zebrafish. Plos One 2012; 7(6) e39327.
30) Su Y-x, Benedek GA, Sieg P, Liao G-q, Dendorfer A, *et al*. Radioprotective Effect of Lidocaine on Neurotransmitter Agonist-Induced Secretion in Irradiated Salivary Glands. PLoS ONE 2013; 8(3): e60256. doi:10.1371/journal.pone.0060256.