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Cao, Yangpei

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2014

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UNIVERSITY OF CALIFORNIA

Los Angeles

Senescing Effects of Ionizing Radiation on Dental Pulp Cells

A thesis submitted in partial satisfaction
of the requirements of the degree Master of Science
in Oral Biology

by

Yangpei Cao

2014

ABSTRACT OF THESIS

Senescing Effects of Ionizing Radiation on Dental Pulp Cells

by

Yangpei Cao

Master of Science in Oral Biology

University of California, Los Angeles, 2014

Professor Mo Kwan Kang, Chair

It was hypothesized that IR triggered premature senescence in dental pulp, resulting in loss of mineralization potential and enhanced expression of senescence-associated inflammation. It was found that IR induced premature senescence and phenotypic alterations in DPSCs in terms of mineralization potential and inflammatory response. Furthermore, IR also led to DNA damage in the dental pulp cells *in vivo*. It was demonstrated that the

application of Yel2, a promising radioprotector, mitigated IR induced premature senescence and DNA damage. These findings elucidated the molecular mechanism by which dental pulp cells responded to IR and may have potentials in advancing therapies for irradiated patients.

The thesis of Yangpei Cao is approved.

Reuben Kim

Shane N. White

Mo Kwan Kang, Chair

University of California, Los Angeles

2014

DEDICATION

I dedicate this work to my family and dear friends for their support.

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ACKNOWLEDGMENTS

First and foremost, I would like to thank my research mentor, Dr. Mo Kwan Kang. I am very grateful to him for giving me lots of research opportunities to explore, for his guidance through every stage of my research projects, for his dedication to education and the genuine concerns for the best interest of his students. I take Dr. Kang as an exemplary scientist and clinician, and keep the greatest admiration to him. I also appreciate Dr. Reuben Kim and Dr. Ki-Hyuk Shin's support for my research in our lab. I am very grateful to Dr. Shane N. White for his input in the manuscript revision. I would like to thank Dr. Wei Chen, Dr. Shebli Mehrazarin, Dr. Jin Kyu Yi, Terresa Kim, Cindy Lee for their kind help with my projects. Also, I thank William Cage from Division of Laboratory Animal Medicine, Dr. Ewa Micewicz and Dr. Kei Iwamoto from Department of Radiation Oncology for their generous help. Finally, I would like to thank all the great friends in our lab for your support and the time we spent together.

INTRODUCTION

A. Radiation: Clinical Effects

Utilization of ionizing radiation (IR) in treating head and neck squamous cell carcinoma (HNSCC) is a well-established treatment method (Brown *et al.*, 2012). Radiotherapy -fractionation regimen was recommended as 70 Gray (Gy) in 35 fractions over 7 weeks (Bonner, 2006). Besides the desired antitumor effects, head and neck radiation therapy (RT) causes side effects in the oral cavity, including xerostomia, radiation caries, taste loss, trismus, and osteoradionecrosis (Vissink *et al.*, 2003). As the 5-year survival rate for patients with HNSCC is about 80% at the early stages and 30-35% with locally advanced stages (Bourhis *et al.* 2006), preserving normal dental tissue function and improving the quality of life should also be considered during treatment.

i) The effect of IR on Tooth Development

Childhood cancer survivors may experience odontogenic developmental abnormalities after exposure to IR (Kaste *et al.*, 2009). Developmental abnormalities include tooth and root agenesis or hypoplasia, delayed eruption, and excessive caries (Thompson *et al.*, 2012). The patients' responses to RT are greatly dependent on the stages of dental development and the radiation factors. RT could arrest tooth bud development if radiation exposure occurs early. Also, radiation may impair differentiation of odontogenic cells or further growth at later developmental and tooth eruption stages (Kimeldorf *et al.*, 1963).

ii) The effect of IR on Salivary Gland

Salivary glands respond to RT acutely as hyposalivation and altered salivary composition (Vissink *et al.*, 2003). Patients reported the feeling of dryness after exposure to 10 Gy (Anneroth *et al.*, 1985). Markitziu *et al.* (1992) evaluated salivary functions of 14 head and neck irradiated patients for a period of 5 years. Whole saliva flow rate (WSFR) was reduced by 96% in patients exposed to 60 Gy or higher during the first year. The irradiated salivary glands possessed severely disorganized sialographic morphology. Dreyer *et al.* (1989) found that the damage to salivary glands was relatively reversible at the dose lower than 30 Gy in a human post-mortem study. However, accumulation of dose greater than 75 Gy caused extensive degeneration of acini accompanied with inflammation, fibrosis in interstitial tissues. These alterations in salivary glands predispose to caries and periodontal disease, impairment of taste acuity, decreased tolerance of prosthetic restorations (Beumer *et al.*, 1978).

B. The effect of IR on Dental Pulp

The changes caused by IR may predispose dental pulp to infection. It was suggested by Silverman and Chierici (1965) that irradiated teeth showed a marked decrease in vascular elements accompanying with fibrosis in the pulp, compromising the pulpal response to infection, trauma and dental procedures. Anneroth *et al.* (1985) examined teeth from patients irradiated for management of HNSCC and found most of the affected teeth suffered varying degrees of pulpitis, although it might be due to decreased salivary production and a change in

food habits. Hommez *et al.* (2008) also reported that apical periodontitis was found in 7.8% of 709 teeth scored in head and neck irradiated patients. Furthermore, Hommez *et al.* (2012) reported that the increased radiation dose led to a higher incidence of apical periodontitis in irradiated patients, but tooth decay was present in 88.2% of teeth. Therefore, the clinical effects of RT on dental pulp are not clear yet.

Researchers also reported findings regarding the effect of IR on dental pulp in various animal models. Hutton *et al.* (1974) studied the effect of cobalt-60 with the dose range of 30 to 70 Gy on dental pulp of two young adult monkeys and could not detect any pathosis in the irradiated pulp. Nickens *et al.* (1977) also reported that cobalt-60 had no effect on the dental pulp of teeth restored with crown or amalgam, composite resin.

Subsequently, Matson *et al.* (1978) irradiated three adult young monkeys with a therapeutic dose up to 70 Gy. Some irradiated teeth were accessed and left open to induce periapical lesions and were endodontically treated. It was concluded that IR caused no difference in the response of dental pulp to endodontic therapy, although higher incidence of fibrous healing was observed in irradiated group. Fawzi *et al.* (1985) further examined the effect of IR on the response of dental pulp to dental procedures by irradiating rats at 4 Gy from a cesium source. They found that IR caused significant depression in the response of exposed dental pulp to infection. However, no significant difference was detected between the irradiated group and the control group if the teeth were left untreated or operated without any pulp exposure.

Decades later, Vier-Pelisser *et al.* (2007) evaluated the effect of fractionated RT on dental pulp of rats under light microscopy (LM) and transmission electron microscopy (TEM). Although nuclear alterations found in the irradiated group on Day 0 were more obvious than those in irradiated group at Day 30, no statistically significant differences were found between the irradiated and control groups in terms of nuclear alteration, matrix hyalinization and inflammation.

According to above clinical findings and animal studies, the adverse effects caused by RT on dental pulp have not been well defined. Further research is needed to resolve the discrepancies among the studies regarding the effects of IR on dental pulp.

C. Senescing effects of IR on Cells

IR induces DNA double-strand break (DSB), further activating signaling pathways for arrest of cell cycle and repair of DNA damage, or cell death if the damage is irreparable (Khanna and Stephen, 2001). The responses of cells to IR are dependent on cell types. It may predominantly involve apoptosis, as studied by Meng *et al.* (2003) in hematopoietic stem cell alike cells, or premature senescence in mesenchymal stem cells isolated from bone marrow (Cmielova *et al.*, 2012) and normal human keratinocytes (Dong *et al.*, 2011). Senescence could occur after replicative exhaustion by shortening of telomere length (Harley *et al.*, 1990) or loss of telomerase activity (Kang *et al.*, 1998). Senescence could also be induced by stresses, such as IR, through a process known as stress-induced premature senescence (SIPS) (Toussaint *et al.*,

2000). Senescent cells also secrete specific, conserved core of up-regulated and unchanged secreted molecules, such as up-regulation of interleukin-6 (IL-6) and IL-8 (Coppe' *et al.*, 2010). These altered secretory activities in response to senescence are known as senescence-associated secretory phenotype (SASP) (Coppe' *et al.*, 2010).

Dental pulp stem cells (DPSCs) were isolated from human adult dental pulp by Gronthos *et al.* (2000). DPSCs possess stem cell-like properties, which could self-renew and differentiate into dentin-pulp like tissues, adipocytes and neuron-like cells (Gronthos *et al.*, 2002). Muthna *et al.* (2010) studied the effects of IR on DPSCs and found DPSCs were arrested in the G2 phase as a result of SIPS. The hallmarks of premature senescence in DPSCs were up-regulation of p21 (inhibitor of cyclin-dependent kinase 1A), p16 (inhibitor of cyclin-dependent kinase 2A) and enhanced activity of SA- β -galactosidase. Thus far, nothing is known about functional characteristics of DPSC after exposure to IR. Premature senescent DPSCs may have impaired stem cell-like properties and induced inflammatory phenotype. The alterations induced by IR could accumulate in dental pulp tissues and further compromise the healing potential of pulp after RT.

D. Hypothesis

RT is frequently used to treat head and neck cancer. However, we are still not clear about the effect of IR on dental pulp. Understanding the mechanism by which DPSCs respond to IR would help us define the pathosis in the dental pulp caused by IR, further advancing

therapies for head and neck irradiated patients. It was hypothesized that IR triggered premature senescence in dental pulp cells, resulting in loss of mineralization potential and enhanced expression of senescence-associated inflammation. To address this hypothesis, we propose following specific aims:

- 1) To investigate if IR induces premature senescence in DPSCs.
- 2) To characterize the phenotypic alterations of DPSCs exposed to IR.
- 3) To determine the effects of IR in pulp cells *in vivo*.

MATERIALS AND METHODS

Cells and cell culture. Primary DPSC cultures were isolated from discarded third molars of adults (19–23 years of age) under institutional review board (IRB) at UCLA, according to the methods described elsewhere (Akiyama *et al.*, 2012). Briefly, extracted teeth were separated along cemento-enamel junction, and the pulp was removed from pulp chamber & root with a dental explorer. Isolated pulp was treated with 2mg/ml collagenase (Sevapharma, Praha, Czech Republic) and 4mg/ml dispase (Invitrogen, Carlsbad, CA) for 1 hour. A cell pellet was obtained after filtration through a 100 μ cell strainer (BD Falcon, Erembodegem, Belgium), centrifugation and re-suspended in α -Minimum Essential Medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 5 mg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA). Normal human oral fibroblasts (NHOF) were prepared from discarded gingival tissues according to the methods described elsewhere (Kang *et al.*, 1998), guided by the UCLA Medical IRB. Cells were cultured in 5% CO₂ atmosphere under 37°C.

Ionizing radiation exposure. Early passage DPSCs (2nd–4th) were seeded in 100mm or 35mm tissue culture dishes (BD Biosciences, San Jose, CA). DPSCs at 70% confluence were irradiated at 5 Gy using a cesium-137 source at the dose rate of 1.743 Gy/min. NHOF were irradiated from the same irradiator at 5 Gy.

Chemicals. Yel2 (Rad2) was kindly provided by Dr. Robert H. Schiestl (David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA). 5 μ M or 10

μM of Yel2 dissolved in DMSO was added to DPSC culture dishes at the time of irradiation.

The culture medium for DPSCs was changed every day with fresh Yel2.

Alkaline phosphatase (ALP) activity and staining. Calcifying condition of DPSCs was induced in the induction medium, which was α -MEM added with 10% FBS, 100 mmol/L ascorbic acid 2-phosphate (Sigma, St Louis, MO), 1.8 mmol/L KH_2PO_4 , 10 mmol/L β -glycerolphosphate, and 9.8 nmol/L dexamethasone (Sigma). 7 days after confluence, cells were lysed in lysis buffer (50 mM Tris-HCl pH = 10, 0.5% Triton X-100, and 1% NP- 40). The lysed cells were mixed with reaction mixture containing 0.2 M glycine-NaOH buffer (pH = 10.4), 16 mM p-NPP, 1 M MgCl_2 and 1 M ZnCl_2 . ALP activity was read at 405 nm and determined on the basis of protein concentration. Also, DPSCs were stained for ALP activity by using an ALP Staining Kit (Sigma) as instructed by the manufacturer.

Alizarin Red S Staining. Confluent DPSCs were induced in the induction medium for 3 weeks. The cells were washed with PBS and then fixed with ice-cold 70% ethanol for 1 hour. Cells were rinsed with double distilled H_2O (ddH_2O) and then stained with 40 mM Alizarin Red S staining solution (pH= 4.2) for 20 min with rotation. Cells were rinsed five times with ddH_2O and photographed with a camera.

Proliferation assay. Proliferation of DPSCs irradiated at 5 Gy and negative control were compared *in vitro*. Starting from Day 0, 1.0×10^5 cells were added to 35mm tissue culture dish. Cells in the irradiated group and control group were counted on Day 2, 4, 6, 8, 10, 12,

and 14. DPSCs were trypsinized for 3 minutes in an incubator and neutralized with 2-fold amount of α -MEM supplemented with 10% fetal bovine serum. Cells were re-suspended in α -MEM after centrifugation and then counted by adding 10 μ l of solution in a hemocytometer. Average counts of duplicated samples were used for proliferation assay.

Senescence-associate β -galactosidase staining (SA β -gal). SA β -gal staining was performed as previously described (Dimri *et al.*, 1995). In brief, DPSCs were washed in PBS, fixed for 4 min in 2 % formaldehyde/ 0.2% glutaraldehyde, and washed again in PBS. DPSCs were incubated overnight at 37 °C (in the absence of CO₂) with fresh SA- β -gal staining solution composed of 20 mg/ml X-Gal in dimethylformamide, 0.2 M citric acid/ sodium phosphate (pH = 6.0), 100 mM potassium ferrocyanide, 100 mM potassium ferricyanide, 5 M sodium chloride and 1 M magnesium chloride. Next morning, cells were washed with PBS and photographed using an Olympus DP72 microscope (Olympus, Tokyo, Japan). Cells with blue perinuclear staining were counted as positively stained cells. The average number of positively stained cells from three random views under microscopy was used for analysis.

Western Blotting. Whole cell extracts were isolated from cultured cells by using lysis buffer (1% Triton X-100, 20 mM Tris-HCl pH = 7.5, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 2.5mM sodium pyrophosphate, 1 μ M β -glycerophosphate, 1mM sodium orthovanadate, 1mg/ml PMSFF), fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon protein membrane (Millipore, Billerica, MA).

The following primary antibodies against phosphorylated-p38 (T180/Y182) (Cell Signaling Technology, Danvers, MA), p38 (Cell Signaling Technology), phosphorylated-p53 (Ser 15) (Cell Signaling Technology), p53 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Chk2 (Thr 68) (Cell Signaling Technology), β -actin (Santa Cruz Biotechnology) were used. The membrane was incubated with primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology), then exposed to chemiluminescence signal.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from irradiated DPSCs and negative control using Trizol™ reagent (Invitrogen Corp., Carlsbad, CA). cDNA was synthesized from 5 μ g RNA as described by Kang et al. (2004). RT-PCR was performed in triplicates with LightCycler 480 master (Roche, Indianapolis, IN) for the relative mRNA expression of interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). The sequences of primers will be available on request.

Tissue preparation. The animal protocol was reviewed and approved by the Animal Research Committee and experiments were carried out as guided by the Division of Laboratory Animal Medicine (DLAM) at UCLA. 8 weeks old C57BL/6 mice (Charles River Laboratory, San Diego, CA) were anesthetized and exposed to total body irradiation of 5 Gy. Irradiated mice were sacrificed with CO₂ tank 1 hour or 1 week after exposure to IR. Non-irradiated mice were served as a negative control. The lower jaws of mice were dissected, fixed, demineralized with 5% EDTA, 4% sucrose in PBS for 2 weeks, embedded

in paraffin blocks and then sectioned to a thickness of 4 μ m. Sections were stained with hematoxylin-eosin (HE) and then observed under light microscopy (LM).

Indirect *in situ* immunostaining. The sections were deparaffined in an oven at 60 °C for 30 minutes, then rehydrated through xylene and series of Ethanol solutions. To eliminate endogenous peroxidase activity, sections were incubated in 3% hydrogen peroxide in methanol for 15 minutes. The sections were repeatedly washed with water and incubated in PBST for 5 minutes. Non-specific binding of antibodies was blocked by 10% locking buffer for 30 minutes. Tissues sections were incubated at 4 °C overnight in a humid chamber with specific anti-P53 monoclonal antibody (1:200 dilution; Cell Signaling Technology). Anti-mouse IgG (1:200, Vectors Laboratories Inc., Burlingame, CA) was used as the secondary antibody, and horseradish peroxidase-avidin (Vectors Laboratories Inc.) were used for third incubation. Tissue sections were washed 3 times with PBST for 5 minutes each between incubations. Sections were developed with DAB solution (Vectors Laboratories Inc.) for 2-5 minutes and washed with water. Counter stained the sections in hematoxylin, dehydrated the slides by going through series of ethanol solutions and xylene and mounted the slides for observation and photography under LM.

RESULTS

IR induces premature senescence in DPSCs. To investigate the senescing effects of IR, early passage DPSCs were exposed to 5 Gy and then stained with SA- β -galactosidase solution on Day 1, 7, 10, and 13. Non-irradiated DPSCs served as negative controls while the replicative senescent DPSCs of passage 26 served as positive controls. The replicative senescent DPSCs had enlarged, flattened appearance and were stained positively for SA- β -galactosidase activity (**Fig. 1a**). Also, IR enhanced the activity of SA- β galactosidase of early passage DPSCs 7 days after irradiation (**Fig.1a**). The induced SA- β -galactosidase activity increased until Day 13 and then slightly decreased on Day 16 (**Fig. 1b**). These data indicated that IR induced premature senescence of DPSCs. To examine the effect of IR on the proliferative ability, proliferation assay was conducted on DPSCs irradiated at 5 Gy and negative controls. The cell numbers of both groups were seeded as 1.0×10^5 on Day 0. Significant inhibition of growth rate was observed in irradiated DPSCs by comparing with negative control (**Fig. 2**). On Day 14, the average cell number of irradiated group was 7.0×10^5 , while the control group has an average cell number of 65.0×10^5 .

IR inhibits differentiation and mineralization potential of DPSCs. To examine the effects of IR on odontoblastic differentiation and mineralization capacities, confluent DPSCs were cultured in induction medium for 3 weeks after exposure to 5 Gy. The results of Alizarin Red

S staining revealed that DPSCs exposed to calcifying conditions were able to form Alizarin Red positive nodules (**Fig. 3**). Irradiated groups were stained less strongly by comparing with negative control, indicating that IR impaired the ability of DPSCs towards odontoblastic differentiation (**Fig. 3**). IR also inhibited ALP activity of irradiated group (**Fig. 4a**), which corresponded to the results of ALP staining (**Fig. 4b**).

IR induces expression of proinflammatory cytokines in DPSCs. To determine the expression of proinflammatory cytokines in response to IR, DPSCs were irradiated at 5 Gy and then compared with negative control on Day 1, 4, 7, 10, and 13. RT-PCR results showed increased expression of interleukin-1 β (IL-1 β) on Day 1 until the peak on Day 10 in a time-dependent manner (**Fig. 5**). Although the expression level of IL-6 remained relatively constant until 10 days after IR, it started to increase on Day 13 (**Fig. 5**). These data indicated that IR might induce the expression of proinflammatory cytokines including IL-1 β and IL-6 in DPSCs.

IR leads to DNA damage in dental pulp cells *in vivo*. To investigate the effects of IR on dental pulp cells *in vivo*, mandibular molars from C57BL/6 mice were sectioned for HE staining and immunostaining. We could not detect significant changes after exposure to IR by HE staining results (**Fig. 6**). However, the immunostaining results suggested that IR induced p53 production in dental pulp cells. In the control group, we did not observe any specifically positive staining of p53 (**Fig. 7a**). However, induction of p53 were detected in the nucleus of dental pulp cells in the sections from mice sacrificed 1 hour after IR (**Fig. 7b**)., Interestingly,

the irradiated group, which was sacrificed 1 week after IR, did not reveal any positive staining for p53 (**Fig. 7c**). These data suggested that IR induced acute DNA damage accompanying with up-regulation of p53 in the dental pulp cells.

Yel2 mitigates IR induced premature senescence in DPSCs. With regards to radioprotection, FDA only approved AmifostineTM as a cytoprotectant for clinical use. However, Amifostine's unfavorable cell toxicity limited its utility (Seed, 2005). A novel radiation mitigator Yel2 was reported to inhibit radiation-induced microhomology-mediated recombination, an error-prone DNA repair pathway contributing to instabilities of genomes (Schiestl *et al.*, 2011). Intriguingly, Yel2 significantly improved the survival rate of C3H mice after exposure to 8 Gy (Schiestl *et al.*, 2013). Therefore, we investigated the effects of IR on irradiated cells in the presence of Yel2 (5 μ m or 10 μ m, dissolved in DMSO). Regarding the staining results of SA- β -galactosidase, we did not detect any significant change caused by Yel2 when compared with DMSO group as the control (**Fig. 8a**). Interestingly, although DPSCs showed some positive stainings at Day 7 after exposure to IR, the irradiated group added with Yel2 did not present any positive staining on Day 0, 4, 7 (**Fig. 8b**). Furthermore, we could not detect any positive staining in the Yel2 group even 30 days after exposure to IR (**Fig. 8c**). These data suggested Yel2 might be able to mitigate IR induced premature senescence in DPSCs.

Yel2 mitigates IR induced DNA damage in NHOFF. We also explored the radioprotective effects of Yel2 in NHOFF. DNA damage markers including phosphorylated checkpoint kinase

2 at Thr 68 (p-Chk2), p-p53, and phosphorylated p38 MAP kinase at Thr180/Tyr182 (p-p38) were significantly induced on Day 2, 4, 8 (**Fig. 9**). In the presence of Yel2, the expression of p-p38 was inhibited on Day 2 and Day 8, while the expression of p-Chk2 and p-P53 were significantly down-regulated on Day 4 and Day 8, suggesting that Yel2 might mitigate IR induced DNA damage in NHOF.

DISCUSSION

RT is a frequently utilized method in treating patients with HNSCC. It is essential to know the adverse effects caused by IR on healthy dental tissues and to elucidate the mechanisms by which dental tissues responded to IR. The contradictory findings regarding the effects of IR on dental pulp warranted further investigation. In the present study, multiple approaches were taken to examine the effect of IR on dental pulp cells *in vitro* and *in vivo*.

It was found that IR induced premature senescence in DPSCs (**Fig. 2**), consistent with previous findings of Muthna *et al.* (2010). However, we furthered our study to explore the phenotypic alterations of DPSCs in response to IR. It was demonstrated that proliferative ability, mineralization potential of premature senescent DPSCs were impaired (**Fig. 1, Fig. 3, Fig. 4**). It is noteworthy that replicative senescent DPSCs also had altered phenotypes including impaired odontogenic differentiation (Mehrazarin *et al.*, 2011). DPSC is characteristic of self-renewal ability and multi-lineage differentiation potential (Nakashima *et al.*, 2013). However, IR, as a stress trigger, altered the functional characteristics of DPSCs, which might explain the reason why IR increased the risks of odontogenic developmental abnormalities.

Interestingly, IR also induced the expression of proinflammatory cytokine IL-1 β and IL-6 (**Fig. 5**) in a time-dependent manner related with IR induced premature senescent activity of DPSCs. IL-1 β constituted most bone-resorptive activity in periapical lesion

(Stashenko *et al.*, 1988). IL-6 was also involved in the adaptive response of symptomatic dental pulp including B cell proliferation (Hahn and Frederick, 2007). Up-regulation of these proinflammatory cytokines after exposure to IR indicated that IR could induce senescence-associated secretory phenotype in DPSCs, although it was not clear if the immune response of dental pulp to infection, trauma or various dental procedures would be compromised.

Subsequently, the study was furthered to investigate the effects of IR on dental pulp cells *in vivo*. We conducted HE staining of tissue sections from mandibular molars of mice irradiated at 5 Gy and could not detect any significant alterations by comparing with control group (**Fig. 6**). It might explain why previous studies (Huttons *et al.*, 1974; Nickens *et al.*, 1977; Matson *et al.*, 1978; Fawzi *et al.*, 1978; Vier-Pelisser *et al.*, 2007) stated that IR caused no difference in the response of dental pulp. However, it was demonstrated that the expression of p53 was induced in dental pulp cells 1h after IR, while positive staining could be found neither in the group sacrificed 1 week after exposure to IR nor in the control group (**Fig. 7**). In response to DNA damage, the p53 tumor suppressor protein orchestrates either arrest of cell cycle temporarily to allow DNA to repair, or the trigger of premature senescence or apoptosis (Le *et al.*, 2010). Our results were supported by the findings of Kastan *et al.* (1991) that the level of p53 in ML-1 myeloblastic leukemia cells increased significantly within 1 hour after gamma irradiation and returned towards normal levels about 48 hours after. The transient change of p53 also correlated well with cell cycle changes

(Kastan *et al.*, 1991), indicating that p53 was involved in the premature senescence of DPSCs induced by IR.

With regards to radioprotection, Yel2 was applied to DPSCs at the time of irradiation. It was observed that Yel2 significantly inhibited SA- β galactosidase activity of DPSCs after exposure to IR on Day 7 and Day 30 (**Fig. 8**), suggesting that Yel2 might have protective effects against IR induced premature senescence. Furthermore, it was investigated if Yel2 could also serve as radiation mitigator in other cells. For NHOF irradiated at 5Gy, the expression of p-p38 was inhibited on Day 2 and Day 8, while the expression of p-Chk2 and p-p53 were significantly down-regulated on Day 4 and Day 8 in the presence of Yel2 (**Fig. 9**). It is noteworthy that the activation of downstream effector kinase Chk2, which was phosphorylated on threonine 68, (Reinhardt and Yaffe, 2009) involved in p53-independent mechanism to regulate the cell cycle. Activated Chk2 could also phosphorylate p53 protein directly by ataxia-telangiectasia mutated (ATM) (Poehlmann and Roessner, 2010). The cross-linking of the cell cycle regulating pathways suggested that Yel2 might involve both p53-independent and p53-dependent pathways to exert its radioprotective effects. Importantly, Yel2 did not cause any significant changes when compared with negative control, indicating that Yel2 might be a safe radioprotective agent to use.

Overall, it was demonstrated that IR induced premature senescence and phenotypic alterations in DPSCs *in vitro* and DNA damage in dental pulp cells *in vivo*. Furthermore, it

was suggested that radioprotection might involve inhibition of IR induced premature senescence and DNA damage.

FIGURE 1

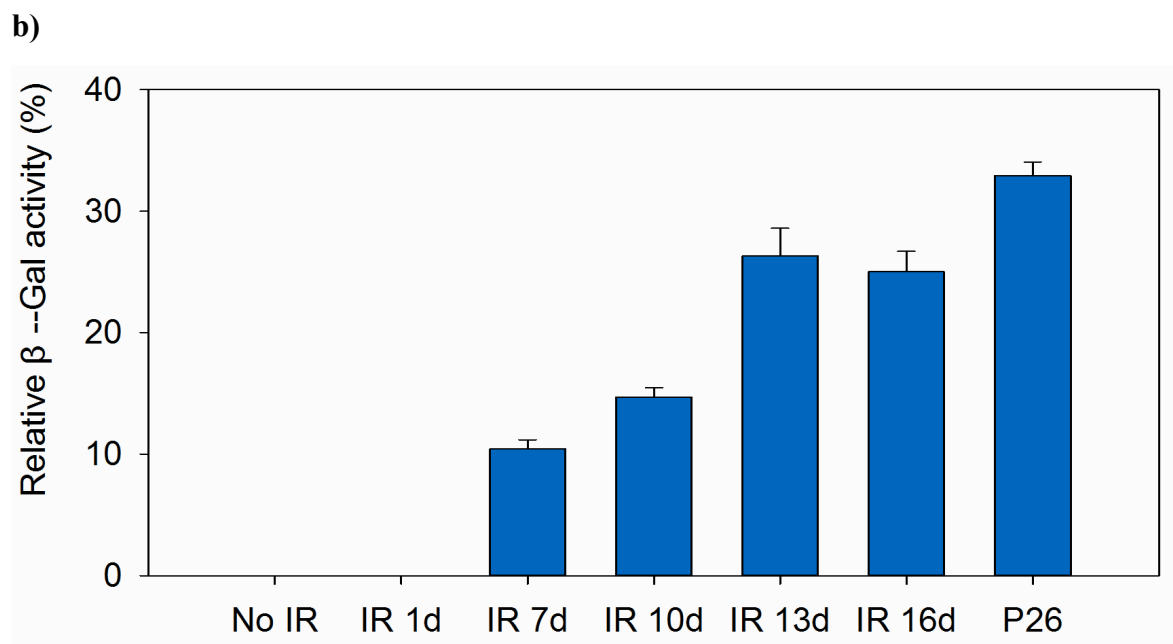
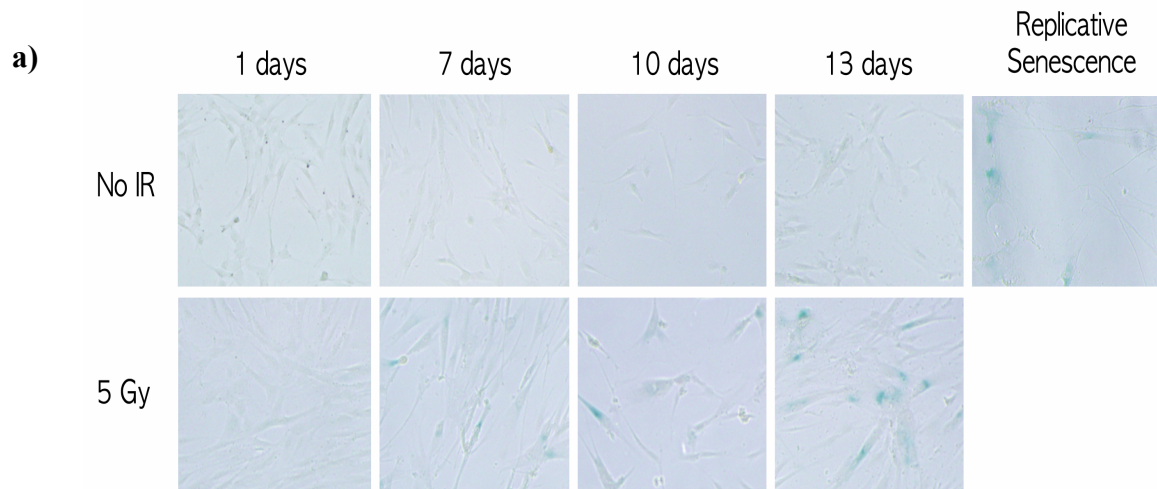


Figure 1- IR induces premature senescence in DPSCs. **a)** DPSCs were exposed to 5 Gy and then stained for SA- β -galactosidase activity 1d, 7d, 10d, 13d, 16d after IR. **b)** Relative positively stained cells were counted and the results were the average values of three random views under light microscopy.

FIGURE 2

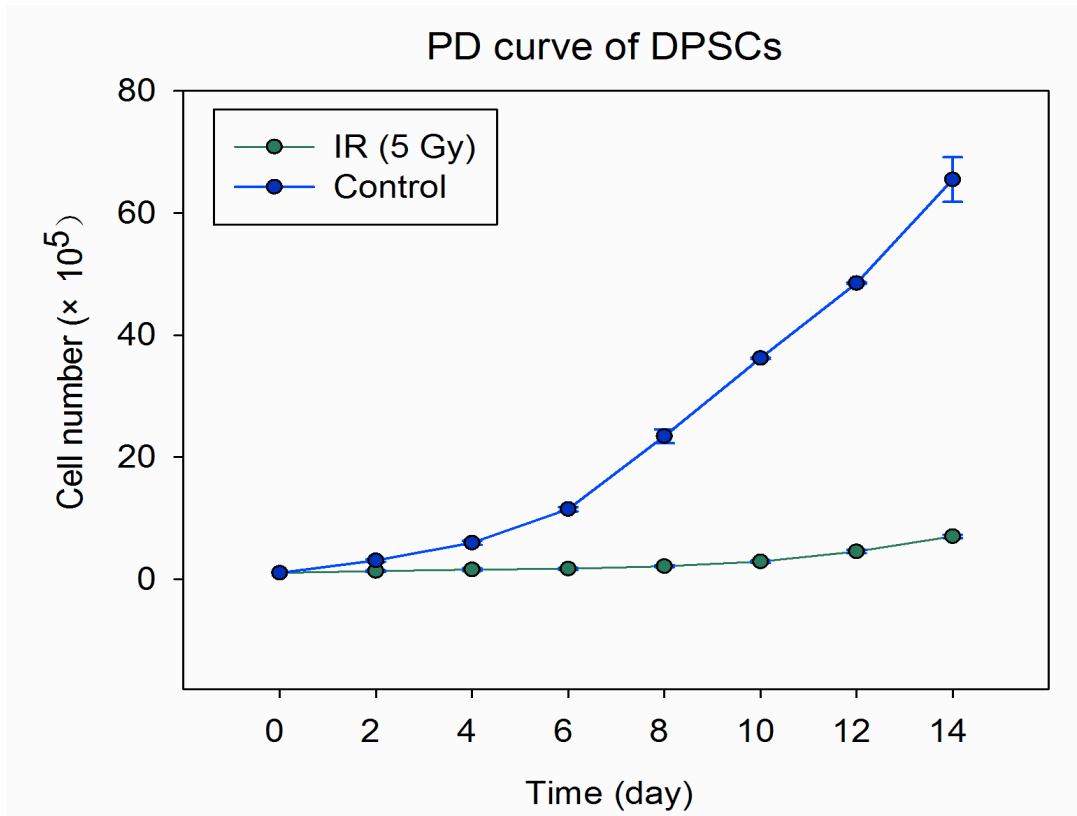


Figure 2 - IR inhibits proliferation of DPSCs. Proliferation assay was conducted on DPSCs irradiated at 5 Gy and compared with non-irradiated DPSCs at Day 0, 2, 4, 6, 8, 10, 12, 14.

FIGURE 3

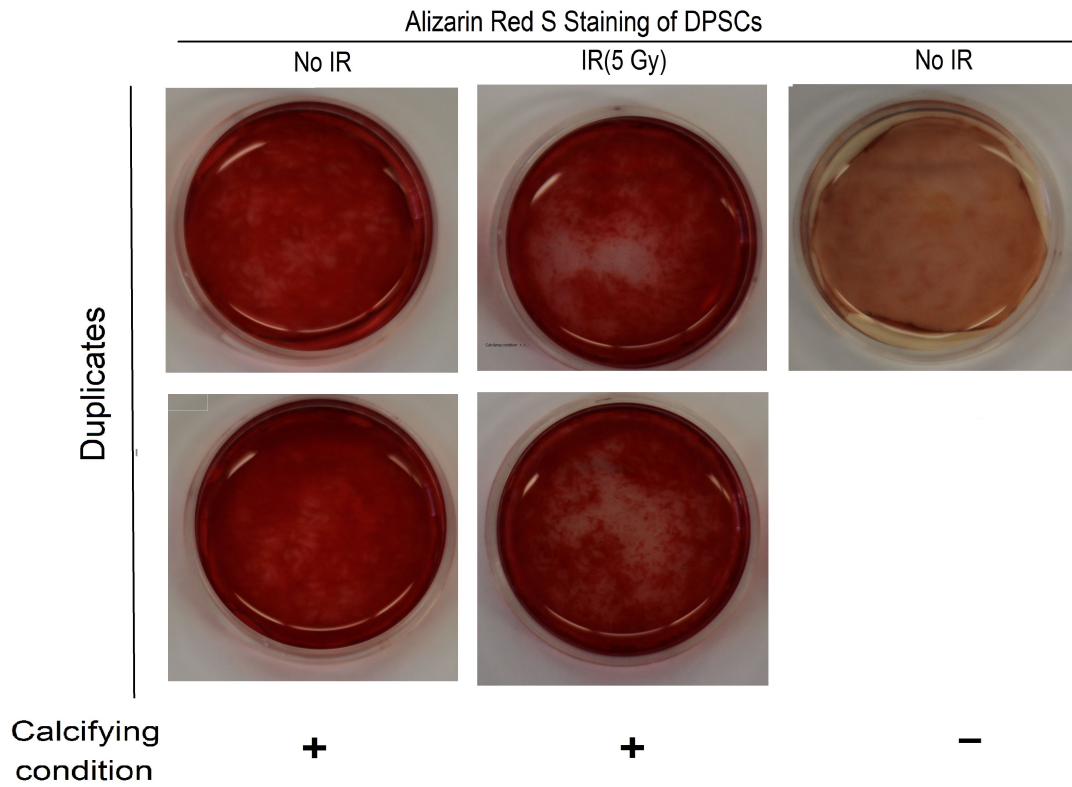
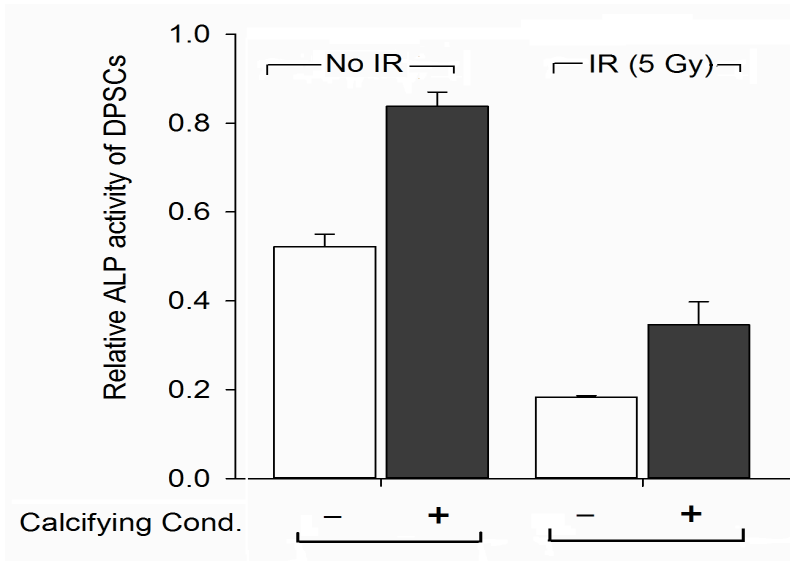


Figure 3 - Alizarin Red S staining of DPSCs in response to IR. Confluent DPSCs irradiated at 5 Gy) and non-irradiated DPSCs were cultured in induction medium (calcifying condition) for 3 weeks. DPSCs cultured in the absence of calcifying condition were served as control.

FIGURE 4

a)



b)

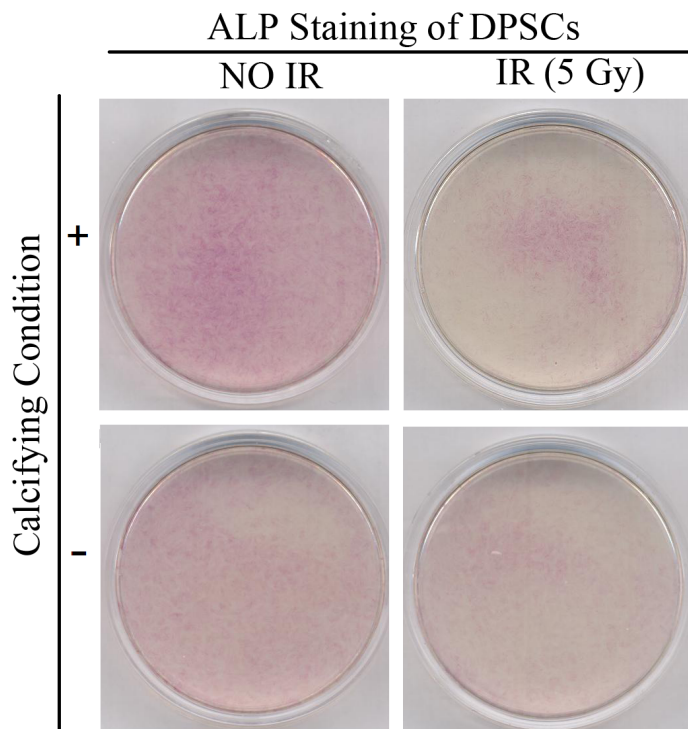


Figure 4 - ALP activity and staining of DPSCs in response to IR. Confluent DPSCs irradiated at 5 Gy and non-irradiated DPSCs were cultured in the induction medium for 1 week. DPSCs cultured in the absence of calcifying condition were served as control.

FIGURE 5

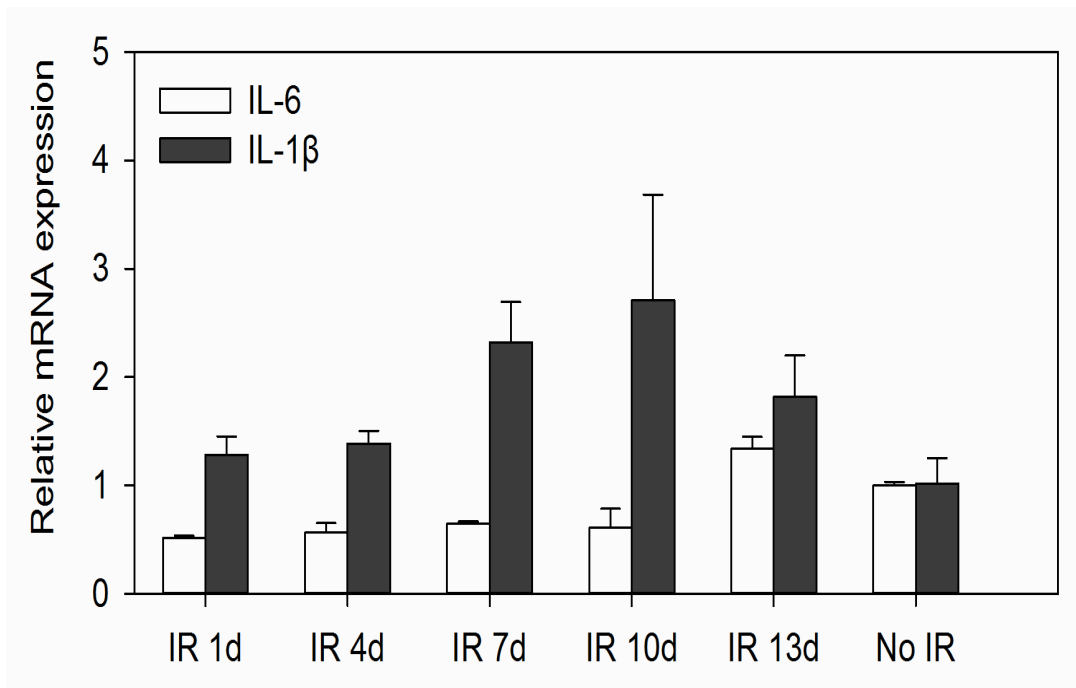
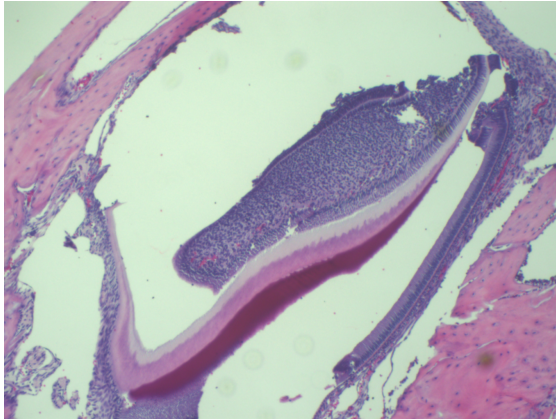


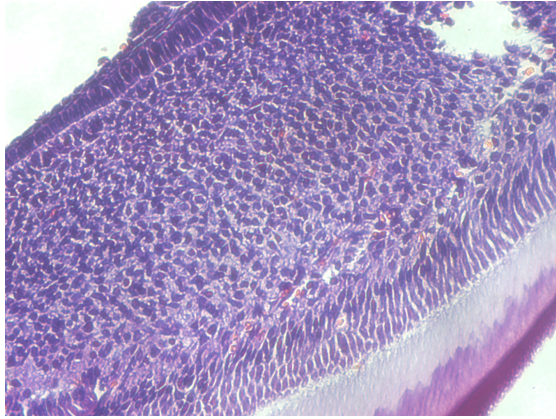
Figure 5 – IR induces expression of IL-1β and IL-6 in DPSCs. The expression levels of IL-1β and IL-6 in DPSCs irradiated at 5 Gy were compared with non-irradiated DPSCs at Day 1, 4, 7, 10, 13, respectively. RT-PCR was performed in triplicate. GAPDH was used to normalize.

FIGURE 6

a)

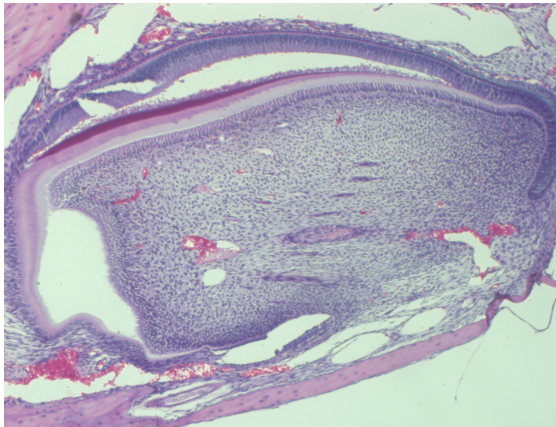


x 100

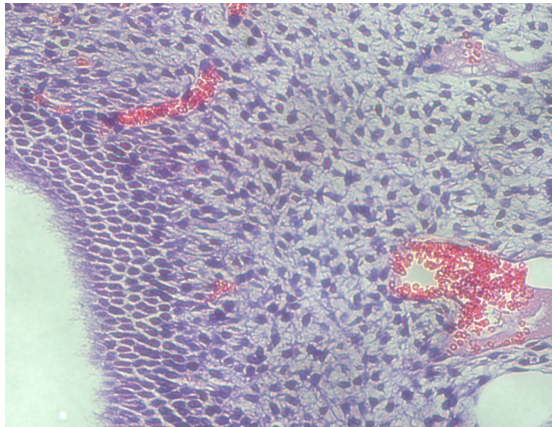


x 400

b)

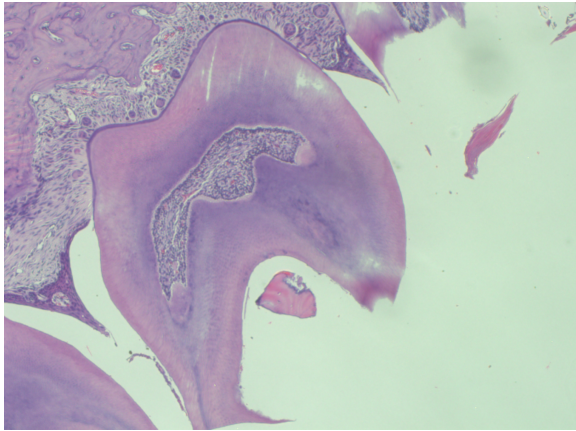


x 100

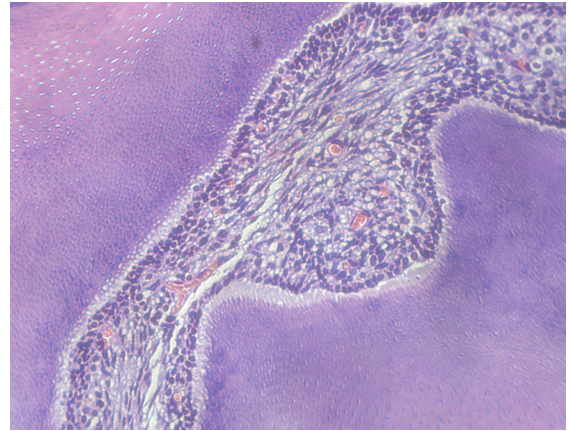


x 400

c)



x 100

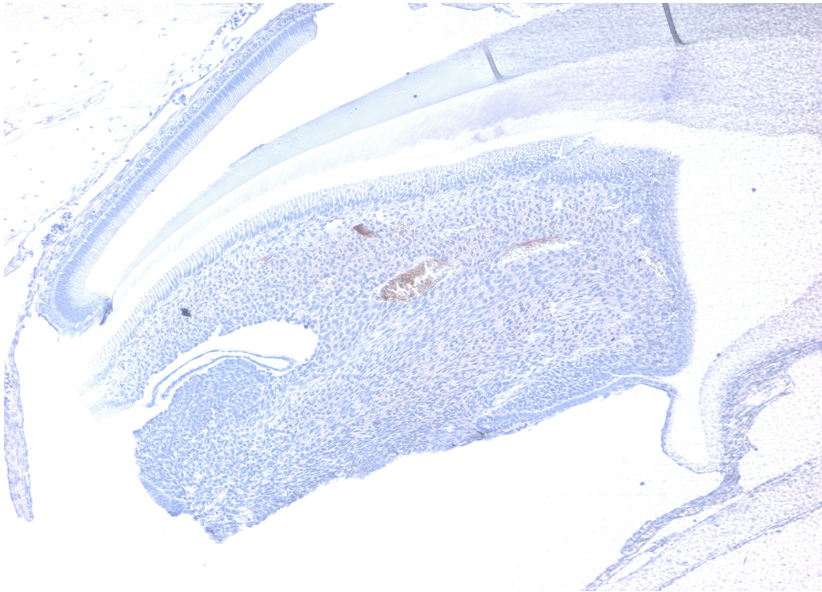


x 400

Figure 6 – HE staining of dental pulp cells in response to IR *in vivo*. HE staining was conducted in the tissue sections of mandibular molar of mice irradiated at 5 Gy. Irradiated groups were sacrificed 1h or 1week after IR, respectively. **a)** HE staining result of control group observed under light microscopy (LM) x 100, x 400, respectively. **b)** HE staining result of irradiated group (1h after IR) observed under LM x 100, x 400, respectively. **c)** HE staining result of irradiated group (1 week after IR) observed under LM x 100, x 400, respectively.

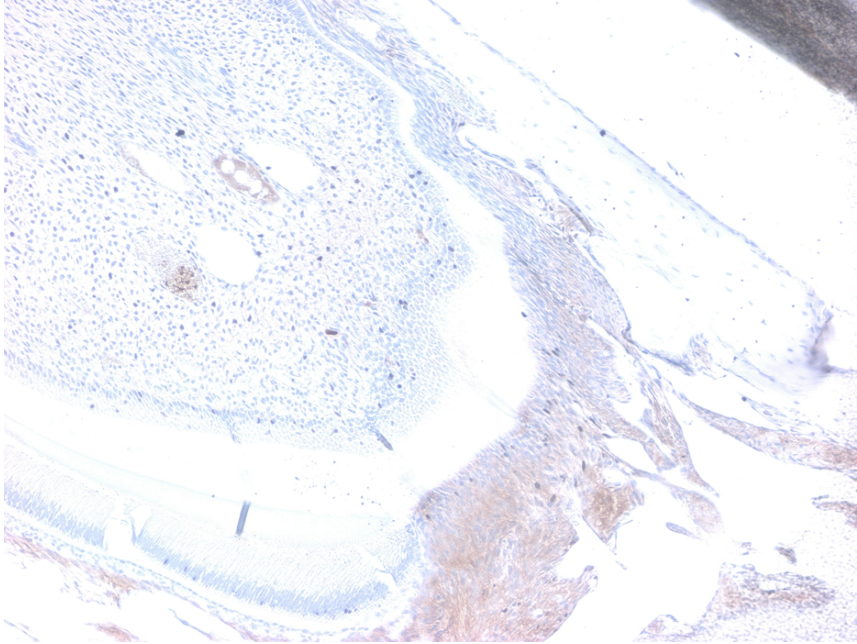
FIGURE 7

a)



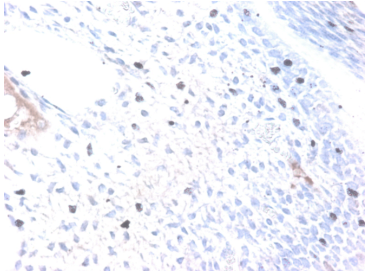
x 100

b)

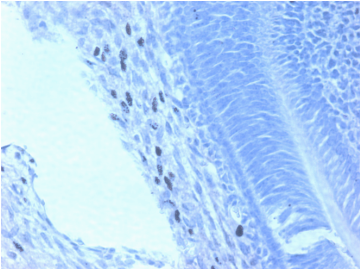


x 100

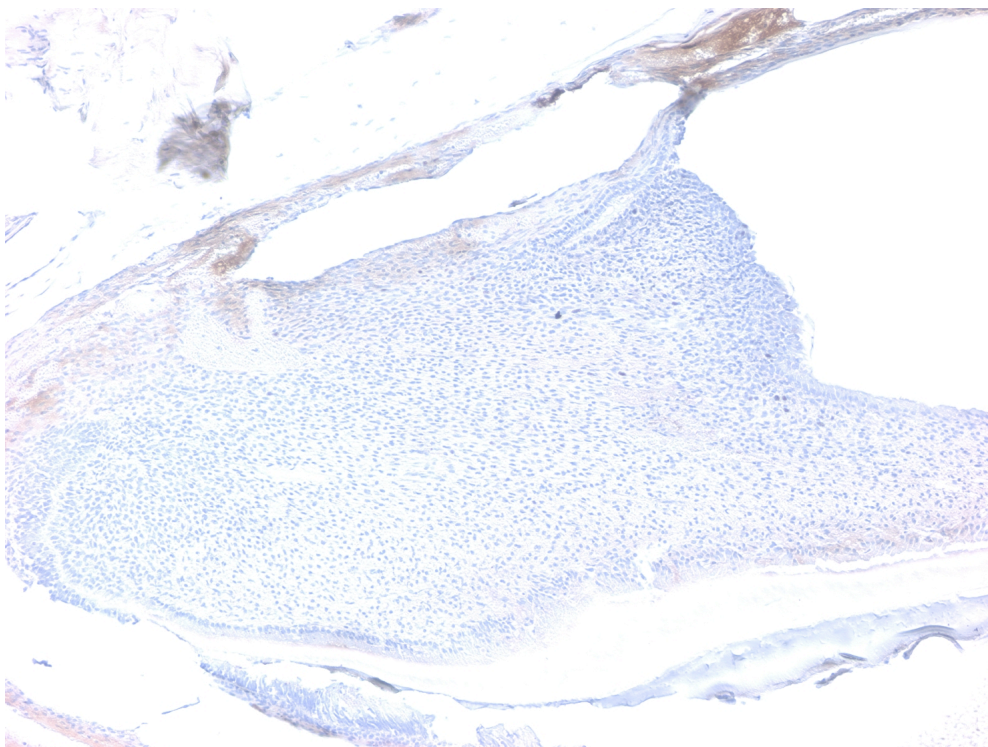
x 100



x 400



c)

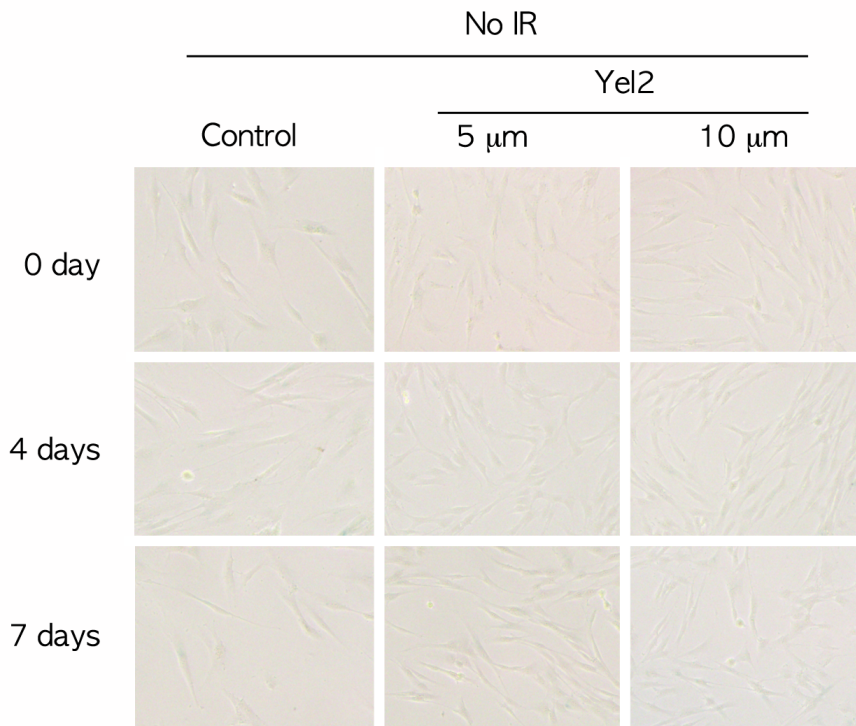


x 100

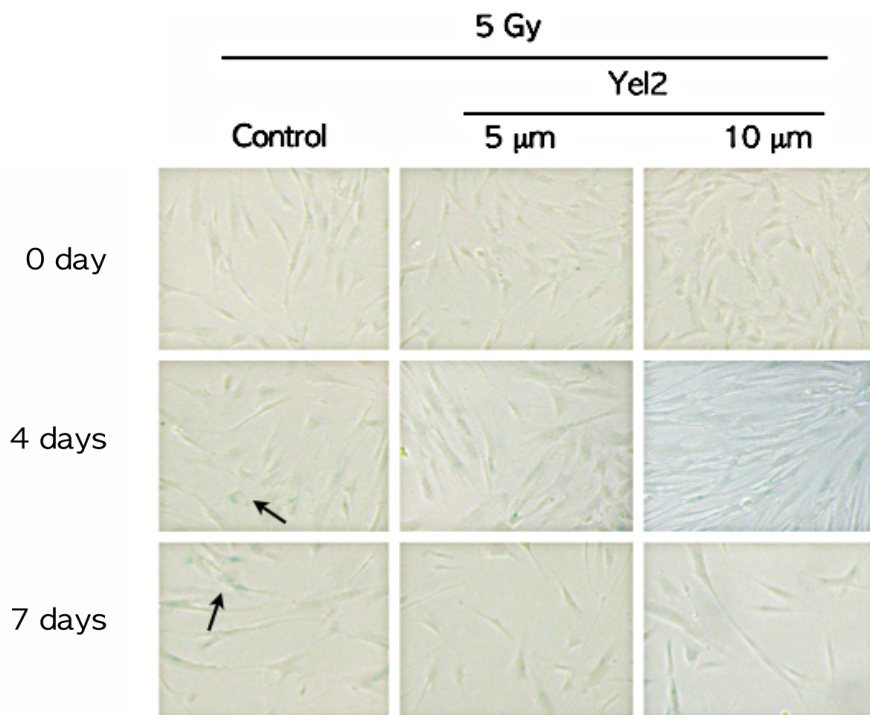
Figure 7 – Immunostaining of dental pulp cells in response to IR *in vivo*. Immunostaining for p53 was conducted in the tissue sections of mandibular molar of mice irradiated at 5 Gy. Irradiated groups were sacrificed 1h or 1week after IR, respectively. **a)** Immunostaining in the control group did not reveal any positive staining observed under LM, x 100. **b)** Nucleus of dental pulp cells in irradiated mice (1h after IR) was stained positively for p53 observed under LM x 100, x 400, respectively. **c)** No positive staining was found in the irradiated group sacrificed 1 week after IR observed under LM, x 100.

FIGURE 8

a)



b)



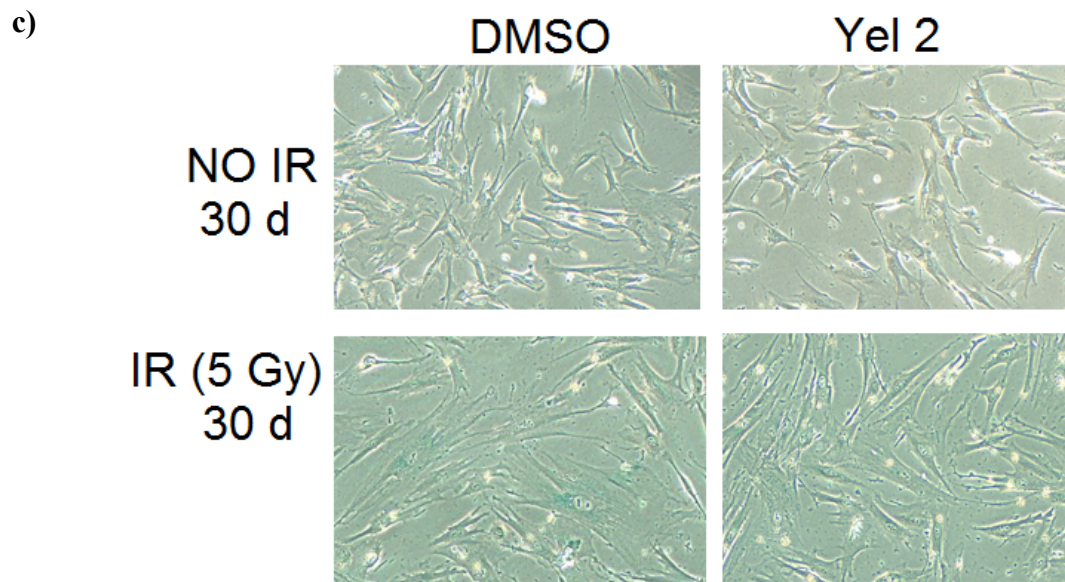


Figure 8 - Yel2 mitigates IR induced premature senescence in DPSCs. a) SA- β -galactosidase activity of DPSCs in the presence of Yel2 (5 μ m or 10 μ m) without IR at Day 0, 4, 7. b) SA- β -galactosidase activity of irradiated DPSCs (5 Gy) in the presence of Yel2 (5 μ m or 10 μ m) at Day 0, 4, 7. c) SA- β -galactosidase activity of irradiated DPSCs with or without Yel2 (5 μ m) 30 days after IR.

FIGURE 9

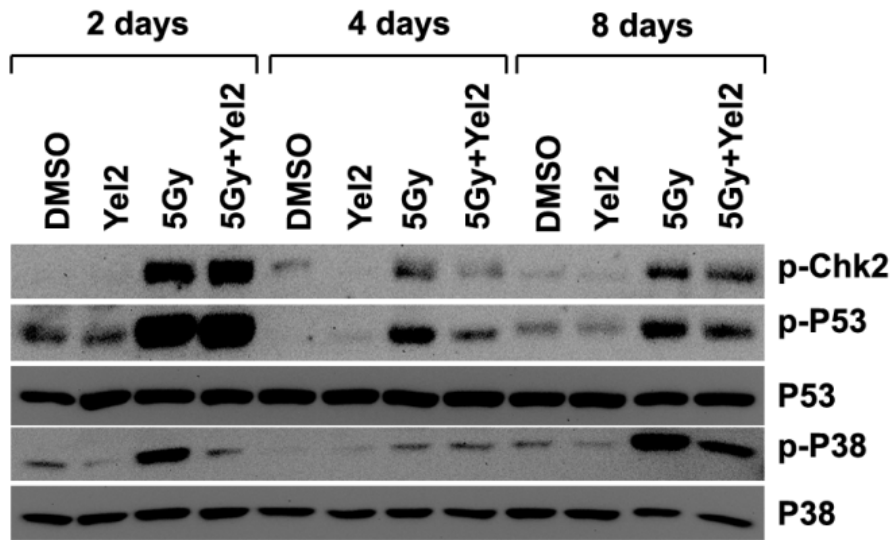


Figure 9 - Yel2 mitigates IR induced DNA damage in NHOF. NHOK were exposed to 5Gy with or without Yel2 (5 μ m) and compared with non-irradiated group at Day 2, 4, 8. Expression levels of phosphorylated Chk2, p53, phosphorylated p53, p38, and phosphorylated p38 were examined by Western Blotting.

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