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ORIGINAL ARTICLE

The Role of the Hippo-YAP/TAZ Signaling Pathway as a Driver of Early Growth Plate Recovery Response to Irradiation

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OBJECTIVE

The growth arrest of epiphyseal plates is an inevitable complication of radiotherapy in most pediatric cancer patients, yet our understanding of the regulatory mechanisms governing the recovery response remains limited. This experiment sought to delve into the roles of yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) transcriptional co-activators, crucial drivers of the regenerative process, in this recovery response.

METHODS

In the study, thirty-six 8-week-old male rabbits underwent radiotherapy targeting the left distal femur and proximal tibia growth plates, while the right extremity served as an unirradiated control. The rabbits were divided into six groups based on post-irradiation time points (1, 7, 10, 14, 17, and 21 days). Histomorphological assessments through hematoxylin-eosin staining and immunohistochemical labeling of PTHrP, YAP1, and TAZ were conducted on the excised growth plates from both irradiated and unirradiated sides.

RESULTS

The initial week post-radiotherapy exhibited arresting effects, including increased apoptosis, reduced proliferative activity, and disruption of the columnar structure. Evidence of the recovery response, characterized by regenerative chondrocyte clones, became evident around day 10 and peaked on day 14. Subsequently, cellular and structural degeneration prevailed, with a transition to osteogenesis in the following days. The radiation notably decreased the nuclear immunopositivity ratio of the three molecules. While nuclear expression levels of YAP1 and TAZ initially declined within the 1st week, they subsequently rebounded sharply, though not reaching control levels.

CONCLUSION

These preliminary findings suggest that YAP1 and TAZ could play a pivotal role as regulators in the recovery response of growth plates following irradiation.

Keywords: Growth plate injury; radiation therapy; recovery response; TAZ; YAP.

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INTRODUCTION

The radiotherapeutic management which is considered a critical constituent of pediatric cancer treatment regimens is the main treatment approach for 30–70% of children who are treated with curative intent.[1] The long-term survival can be achieved in a major part of patients who receive radiotherapy. Unfortunately, almost all these patients are afflicted with radiotherapy-related late toxicities. Radiation-related developmental defects of epiphyseal growth plates, including short stature, angle deformities, and differences in extremity lengths are some of main concerns in patients who have long survival.[2] Depending on the localization of tumor or radiotherapy field (craniospinal irradiation, etc.), growth plates are frequently exposed to low-dose irradiation or sometimes remain directly within high dose area. Unluckily, because growth plates are quite radiosensitive, arrest of epiphyseal bone growth is inescapable even with low doses.

Although the radiosensitivity of growth plates and the adverse impact of radiation on epiphyseal growth well known for a long time, neither the molecular mechanisms have been elicited nor effective protective or treatment methods have yet to be discovered.[3–7] In the past, some non-selective radioprotective drugs including amifostine, pentoxifylline, misoprostol, and selenium were employed to minimize the risk, but the effectiveness of these drugs remained limited, and the trials were unable to go beyond animal experiments.[4,8–11] As another option, the optimization of radiation fields and doses can help alleviate the severity of damage. For that purpose, the growth plates can be excluded from target volumes as much as possible without compromising treatment outcome. In case it is not possible, the doses on the growth plates can be reduced as low as possible, the fractionated regimens can be used, or the acceptable homogenous dose distribution can be delivered.[12] These modifications can prevent the progress of angle deformities such as kyphosis, lordosis, and scoliosis. Nevertheless, in most cases, growth arrest of irradiated plates is not obviated, despite limiting exposure to growth plates.

It is well known that RT causes premature terminal differentiation and leads to the early closure of growth plates by affecting chondrocyte differentiation.[3,5,13,14] However, some studies have instantiated that the retardation effect of radiation on growth plates is temporary and a recovery response in the growth plates emerges after 2nd week.[4,6,7,15–17] But, in the published literature, the mechanisms that direct the recovery response are under-researched. As far as is known, the gene expression of chondrocytes shows significant changes, particularly in proliferative and hypertrophic chondrocytes, during the early stages of the recovery response.[6,7] That gene switch alters growth factor activity, such as PTHrP, which is the main control factor of chondrocyte differentiation, and modulates activity of pathways that regulate bone development, cartilage development, ossification, angiogenesis, and matrix structure. However, it has not yet been investigated how and which factors lead genetic modulation to change as it transitions to the recovery phase.

Hippo signaling pathway is a recently discovered kinase cascade that regulates organ size, cell proliferation and differentiation, stem cell fate determination, and activity of growth factors.[18] In briefly, the cascade takes input from several intracellular and extracellular signal sources and controls the phosphorylation-dependent subcellular localization of yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) transcriptional co-activators. When the activity of cascade reduces, YAP and TAZ are dephosphorylated and then translocate into the nucleus from the cytoplasm. YAP/TAZ which accumulated in nucleus binds to transcription factors (mostly TEAD), and then, this transcriptional complex modulates the transcription activity of various target genes.[19]

At first, hippo-YAP/TAZ signaling pathway had been defined as a main regulator of tissue growth and organ size. But, over the past two decades, the growing body of evidence has shown that the influence of this pathway extends beyond this role. In fact, this pathway also serves as a master regulator of regenerative and repair processes of tissues.[20,21] A few recent researches have revealed the evidence to show that hippo-YAP/TAZ plays a crucial role in endochondral development, as well as its differentiation and regeneration.[22–26] According to one of these studies, the morphogenesis of cartilage is regulated by YAP activity.[22] While in another experimental study, Deng et al.[23] report that increased activity of YAP1 promotes the chondrocyte proliferation, whereas it inhibits maturation and terminal differentiation of chondrocytes. Additionally, the same study has indicated that enhanced YAP activity can be to blame of maladaptive repair response in an experimental fracture model. Although there is currently no evidence linking radiation-related damage to growth plates with hippo-YAP/TAZ signal activity, studies have shown that irradiation can induce this pathway and increase YAP/TAZ activity in other tissues. In parotid salivary gland, induction of YAP nuclear transloca-

tion increased the self-renewal capacity of irradiated salivary gland stem/progenitor cells.[27] Additionally, increased YAP activity following the radiation therapy which causes resistance to treatment has been observed in glial and breast malignant tumors.[28,29]

After the radiation damage, although there is a recovery response as above mentioned, the original morphology of growth plate cannot be fully restored and the growth rate remains under its healthy levels. [17] Therefore, the recovery response seems like an incomplete repair process resulting in bone growth defects rather than a complete restoration of damage. Despite this, the recovery response probably determines the final magnitude of damage in the epiphyseal growth plates following radiation exposure. Elucidating the molecular and genetic regulation of recovery response may provide us with the opportunity to reduce the damage with selective therapeutic interventions. Considering its pivotal role in endochondral development and repair processes, it is not surprising that the hippo-YAP/TAZ signaling pathway could have a prominent role in the recovery response that initiates the regeneration process in the growth plate damaged by ionizing radiation.

The main purpose of our experiment was to explore the role of YAP1/TAZ (the effectors of hippo pathway) in recovery response of growth plates following radiation-related damage. Herein, we reported changes in the nuclear expression levels of YAP1/TAZ (potential master regulators for recovery response) and PTHrP during the early period and the recovery phase after completion of fractionated radiation therapy. Our findings open a new perspective into the radiotherapy-related growth plate damage and early recovery response which can be orchestrated by YAP1/TAZ.

MATERIALS AND METHODS

Animals and Radiation Treatment

The experiments were performed in thirty-six New Zealand white, male rabbits. The age of rabbits was about 8 weeks, and their weight was approximately 1500–2000 gr. The process of research and the experimental procedures on rabbits were approved by the Institutional Local Ethics Committee for Animal Experiments (G.Ü.ET-20.043). During the entire experimental period, the animals were housed in single-seater cages and were fed ad libitum. A controlled 12-h light-dark cycle, temperature, and relative humidity were maintained in the room.

The rabbits were immobilized in the prone position on a vacuum bag under general anesthesia with intramuscular ketamine (45 mg/kg) and xylazine (5 mg/kg) for radiotherapy simulation and treatment. After computed tomography simulation for radiotherapy, the distal growth plate of left femur and the proximal growth plate of left tibia were contoured as target volume. A three-dimensional conformal radiotherapy plan was created with two opposite fields using 6 MV photon energy at source-axis distance of 100 cm. Additionally, 10 mm thick bolus materials were used to achieve proper dose distribution (Fig. 1a). In compliance with clinically relevant model, a total dose of 17.5 Gy was administered in five fractions (3.5 Gy/fraction) on consecutive days. The targeted left growth plates of all animals received radiotherapy and the same growth plates of right extremity served as control groups without radiotherapy. The thirty-six rabbits divided into six groups (all groups had six rabbits) according to the following time. Animals in each group were humanely sacrificed at intervals of 1, 7, 10, 14, 17, and 21 days, respectively, after the last day of irradiation. After then, the growth plates of left and right knees were excised and taken to neutral formaldehyde for histological and immunohistochemical analysis.

Histological and Immunohistochemical Analysis

The obtained tissues were fixed in a 10% formaldehyde solution for a minimum of 72 h. Subsequently, the tissue samples were placed in an appropriate solution for decalcification and left to soak. After achieving decalcification, all tissues were placed in cassettes and washed under running water. To remove water, the tissues were passed through increasing alcohol series (50%, 70%, 80%, 90%, and 100%). Then, the tissues were passed through xylene for clearing and embedded in melted paraffin for sectioning. Sections of 4 μm thickness were obtained from the prepared paraffin blocks. Hematoxylin-eosin (H&E) staining and immunohistochemical labeling for PTHrP, YAP, and TAZ were performed on all samples. Sections of 4 μm thickness were obtained from bone tissue blocks in all experimental groups. After incubating the sections at 60°C in an oven for 1 hour, they were deparaffinized by immersion in xylene for 3×10 min and then rehydrated by passing through decreasing alcohol series (100%, 96%, 80%, and 70%). To remove residual alcohol, the sections were passed twice for 1 min each through distilled water. For antigen retrieval, 1/10 diluted EDTA buffer was applied. After the wash step with distilled water, endogenous peroxidase activity was blocked by

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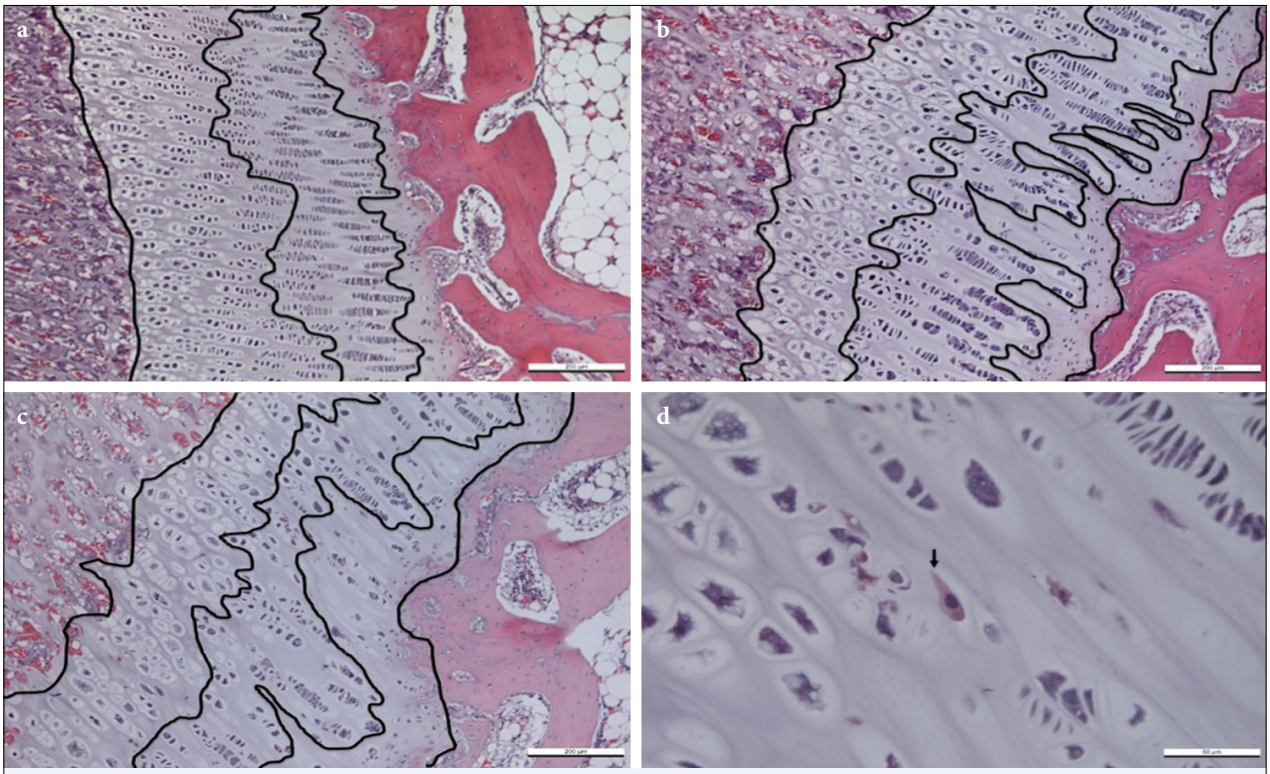

Fig. 1. The representative histomorphological results of the hematoxylin-eosin staining (scale bar: A-C 200 µm, D 50 µm) (different zones of the growth plate are outlined by the black line). (a) A normal organized columnar arranged growth plate. (b) At day 1, irradiated growth plate. (c) At day 7, irradiated growth plate. (d) The down arrow sign (↓): The cells having an increased eosinophilic cytoplasm.

incubating the tissues with 3% hydrogen peroxide for 10 min. The primary antibody was incubated for 2 h in a humid environment, followed by a 30-min incubation with HRP Polymer Quanto. Careful washing with PBS was performed at each step. DAB staining was used to visualize positive cells, followed by a 30-s staining with Harris Hematoxylin and rinsing with running water for 2x1 min. The slides were immersed in a mixture of 1% ammonia water for 1 min and rinsed in running water. After removing excess water, the stained slides were cleared by immersion in xylene for 5 min and then mounted with Entellan. The obtained samples were examined using a Leica DM 4000 (Germany) computer-assisted imaging system and evaluated by capturing images with the Leica-Qwin program.

Statistical Method

The nuclear immunopositivity ratio of YAP1, TAZ, and PTHrP was determined by counting 100 cells. For each molecule, the mean immunopositivity ratio of control growth plates on the 1st day (Group 1) was considered as the nominal "1" value, and the ratios of other days were calculated as fold changes based on this assumption. The Repeated Measures Variance Analysis (ANOVA) test was used to assess changes within days in control and radiotherapy groups. The pairwise comparisons between radiotherapy groups were analyzed with Bonferroni correction of the same test. The Wilcoxon signed-rank test was used the pairwise comparisons between radiotherapy group and control group on the same day. The Spearman's rank correlation test was performed to determine the strength of correlation between the changes of YAP, TAZ, and PTHrP. The statistical differences of data were analyzed with SPSS software (IBM Corp. Version 28.0.1), and the results are presented as mean±standard deviation (SD). A $p<0.05$ was considered statistically significant, for all tests.

RESULTS

Histomorphological Results

In the general histomorphological examination performed with hematoxylin and eosin, the zonal histomorphological changes in the growth plates exposed to radiation therapy were minimal compared to the

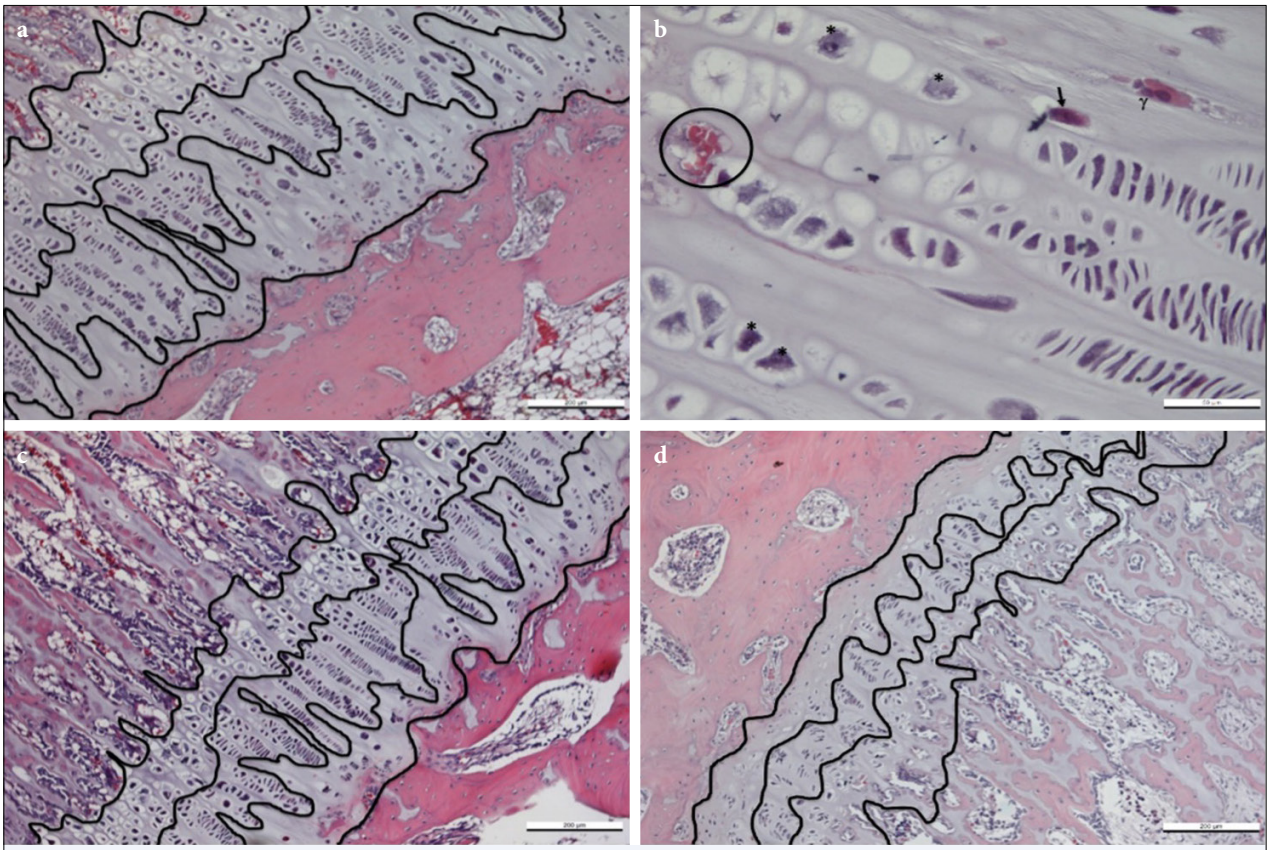

Fig. 2. The representative histomorphological results of the hematoxylin-eosin staining (scale bar: A, C, D 200 μm, B 50 μm) (different zones of the growth plate are outlined by the black line.) (a) At day 10, irradiated growth plate. (b) The down arrow sign (↓): the cells having an increased eosinophilic cytoplasm, the circle sign (O): capillary formation associated with vascular invasion, The gamma sign (γ): chondroblastic cells, The asterisk sign (*): chondrocytes with the cytoplasmic vacuolization. (c) At day 14, irradiated growth plate D: At day 21, irradiated growth plate.

control group at 24 h after fractionated radiotherapy (Fig. 1b) The main finding in this day was increased apoptosis observed in the proliferative and hypertrophic zone areas. Structural changes first occurred in the proliferative zone, and the normal columnar structure of the proliferative zone began to degenerate (Fig. 1b). On this day, the total length of the growth plate was increased in the experimental group compared to the control group. However, in the following days, the length of the growth plates exposed to radiation irreversibly decreased compared to normal growth plates.

The histomorphological changes become frankly prominent in the 1st week after radiotherapy (Fig. 1c). A significant decrease in proliferative activity of chondrocytes was observed in the experimental groups. Further increase of radiotherapy-induced apoptosis accompanied the proliferative decrease. The lengths of hypertrophic and proliferative zones were decreased compared to the control group, whereas the relative increase in matrix area fraction was shown. Cellular structural changes and necrosis also emerged in the same zones. These findings progressively increased in the following days. On this day, the cells having lacunar structures, which are thought to be secondary to increased lysosome content, with eosinophilic cytoplasm compared to other chondrocytes, were detected in the growth plates exposed to radiation (Fig. 1d). The number of these cells increased toward the 17th day.

The matrix area fraction of the hypertrophic zone, which had shown a significant increase on days 7 and 10 after radiotherapy, returned to the normal growth plate level on the 14th day (Fig. 2a). The accompanying increase in the number of chondroblastic cells was noteworthy (Fig. 2b). The emergence of chondroblastic cells, along with the increase in cells with high lysosomal content, indicates an increase in matrix degradation and a decrease in the matrix area fraction secondary to this.

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The regenerative chondrocyte colonies, the indicator of recovery response, started to increase from day 10 and showed the most significant increase on day 14 (Fig. 2c). Proliferation rate and volumetric growth also increased in the RT-exposed plates, secondary to this increase. However, despite this increase, growth rate and volumetric growth in the growth plates exposed to radiation therapy remained consistently lower than the control group throughout the entire experiment.

In the 17th and following 21st days, the length of resorption zone increased and the matrix degeneration in this zone peaked (Fig. 2d). An increase was observed in chondrocytes with the cytoplasmic vacuolization, thought to be secondary to lipid accumulation (Fig. 2b). In the 21st day, the increase in capillary formation associated with vascular invasion, which is a sign of transition to osteogenesis, became prominent (Fig. 2b and d). At the same time, a significant increase in the ratio of bone tissue/cartilage tissue was detected. The chondrocyte cellularity decreased significantly, and necrosis became a major finding accompanying cellular and structural distortion.

Immunohistochemical Results

In control growth plates, the expression of YAP, TAZ, and PTHrP remained relatively stable from the 1st day to the 21st day (p values were 0.1, 0.5, and 0.6, respectively). On the other part, irradiation markedly decreased the nuclear immunopositivity ratio of three molecules in all time points of experiment (p<0.001 for all of them) (Fig. 3).

The nuclear expression level of YAP1 regressed in 1st week and reached its minimum level with 0.46-fold change at the 7th day. At this point, it showed a clear recovery from the minimum level and continued to rise following days. The sharpest rise in expression levels of YAP1 was between the 14th day and 17th day (a 55% increase, p=0.008) (Table 1). Notwithstanding, the levels of expression in the radiation groups did not attain those observed in the control groups. However, by the 21st day after irradiation, expression levels were statistically similar between the control and experimental irradiated groups for YAP1. (p=0.09) On the other hand, the nuclear expression level of TAZ displayed a similar change to what happened in YAP1 (the coefficient of correlation was 0.76, p<0.001). However, there was a slightly smoother change for TAZ.

The expression of PTHrP was at its minimum level with 0.24-fold change on the 1st day differently from YAP1 and TAZ. Following the lowest level, the expression of molecule showed a gradual increase up to the 21st day. The change in the expression levels of PTHrP in growth plates exposed to irradiation showed a strong correlation with each other (p<0.001 for both correlation statistics).

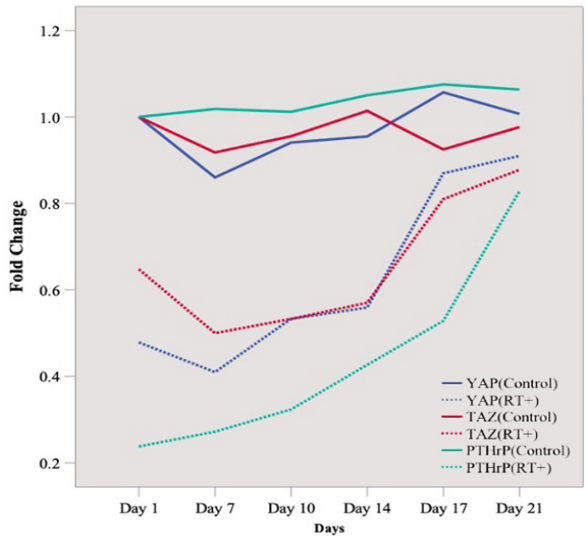

Fig. 3. The comparative graph of changes in the expression levels of YAP1, TAZ, and PTHrP between the irradiated experimental groups and unirradiated control groups (1.0-fold change is the mean value of day 1 control group) (the results of repeated measures analysis of variance test for the comparisons of changes between control and irradiated groups: P<0.001 for YAP1, TAZ and PTHrP).
YAP1: Yes-Associated Protein 1; TAZ: Transcriptional Coactivator with PDZ-binding Motif; PTHrP: Parathyroid Hormon-related Protein.

DISCUSSION

Physeal arrest is a devastating complication of ionizing radiation in children and often culminates in premature closure. Despite its importance, the mechanisms of damage have been not well established. Only a few studies which were conducted by the same group have focused on this issue.[4–11,14,15,17,30–38] Their animal models evinced that the damaged growth plate with irradiation exhibits an early recovery response. However, their great efforts have not provided a clear vision for regulatory mechanism of recovery response. The hippo-YAP/TAZ signaling pathway which is known to play main roles in the regenerative process has been not examined in irradiated growth plates, before. Here, we suggest that the YAP/TAZ activation can be a key regulator and a critical step for

Table 1 The relative changes in PTHrP, YAP1, and TAZ nuclear expressions between days in the irradiated growth plates

	PTHrP		YAP1		TAZ	
	Fold change	**p**	**Fold change**	**p**	**Fold change**	**p**
Control vs Day 1	0.24	0.004	0.48	0.004	0.65	0.004
Control vs Day 7	0.27	0.004	0.46	0.004	0.55	0.004
Control vs Day 10	0.32	0.004	0.57	0.004	0.56	0.004
Control vs Day 14	0.40	0.004	0.59	0.004	0.56	0.004
Control vs Day 17	0.49	0.004	0.82	0.004	0.88	0.01
Control vs Day 21	0.78	0.004	0.90	0.09	0.90	0.146
Day 1 vs Day 7	1.14	1	0.86	1	0.077	0.008
Day 1 vs Day 10	1.36	1	1.12	1	0.82	0.017
Day 1 vs Day 14	1.79	0.002	1.16	1	0.88	0.343
Day 1 vs Day 17	2.2	<0.001	1.82	<0.001	1.24	<0.001
Day 1 vs Day 21	3.48	<0.001	1.9	<0.001	1.35	0.004
Day 7 vs Day 10	1.19	0.816	1.3	0.659	1.07	1
Day 7 vs Day 14	1.57	0.121	1.36	0.882	1.14	1
Day 7 vs Day 17	1.94	<0.001	2.12	<0.001	1.62	<0.001
Day 7 vs Day 21	3.04	<0.001	2.22	<0.001	1.76	<0.001
Day 10 vs Day 14	1.32	0.501	1.03	1	1.07	1
Day 10 vs Day 17	1.63	0.001	1.63	<0.001	1.52	<0.001
Day 10 vs Day 21	2.56	<0.001	1.71	<0.001	1.65	<0.001
Day 14 vs Day 17	1.24	0.021	1.55	0.008	1.42	<0.001
Day 14 vs Day 21	1.94	<0.001	1.62	0.005	1.54	0.002
Day 17 vs Day 21	1.57	<0.001	1.04	1	1.08	1

Mann–Whitney U-test was employed to compare the irradiated groups with control groups. For other comparisons, repeated-measure analysis of variance with Bonferroni correction was utilized. A p-value <0.05 was considered statistically significant. PTHrP: Parathyroid Hormon-related Protein; YAP1: Yes-Associated Protein ; TAZ: Transcriptional Co-activator with PDZ-binding Motif

recovery response. In our rabbit model, the histomorphological results demonstrated that the inhibitory effects of irradiation, including apoptosis, decrease in proliferative activity, and degeneration of columnar structure were evident during the 1st week. The nuclear expression levels of YAP1 and TAZ also dramatically reduced during the same period of observation and reached their lowest point on the 7th day. The appearance of regenerative chondrocyte colonies on the 10th day of experiment has indicated the initiating of recovery response. During the period when clonogenic proliferation and the recovery response occur, nuclear expressions of YAP1 and TAZ also increased by approximately two-fold. The remarkable increase in nuclear expression of these transcriptional activators at the early recovery phase affirms our initial hypothesis which is that YAP/TAZ is the main regulators for compensatory recovery response. But, this regenerative activity in recovery phase fell short of complete prevention of the damage.

Radiation destroys the epiphyseal growth plate structure and affects adversely endochondral differentiation in skeletal immature patients. The histomorphological results of radiation on growth plates are well documented in the above-mentioned previous studies. Our results also showed that the process starting with apoptosis of chondrocytes continues with the destruction of normal columnar structure and eventuates with ossification. The injured growth plate responds to the first depressive effect of radiation with a compensatory proliferative activity. The emergence of regenerative chondrocyte clones is the main sign of this response, which is also called the early recovery response. Unfortunately, a knowledge gap about molecular and driver mechanisms that lie beyond the morphological evidence exists.

In accordance with previous studies, our findings revealed that the initial and most noticeable histological evidence of recovery response were appearance of regenerative chondrocyte colonies and enhanced proliferation rate.[15,17] Unsurprisingly, the increased activity of stimulant molecules such as PTHrP, IGF, and CTGF for chondrocyte proliferation is a necessary step for recovery response.[4,16] PTHrP is the potent proliferative molecule for chondrocytes in growth plates.[39] Therefore, the potential role of PTHrP in

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radiorecovery has been aroused interest.[5,14,40] In a study, Damron et al.[4] observed a remarkably consistent correlation between the pattern of changes in PTHrP expression levels and histomorphological alterations of recovery. According to their report, PTHrP expression reached its lowest level at 1 week after irradiation, but a notable return in PTHrP expression has observed at week 2, coinciding with the appearance of proliferative clones. In a subsequent study conducted by the same research group, the role of PTHrP in recovery response to radiation injury has been once again examined with and without radioprotectant combinations.[37] Depending on the findings, radioprotectants reduced the early drop in PTHrP but also blunted the increased of PTHrP at week 2. However, the proliferation rate assessed by BrdU staining has increased in combination radioprotectants group despite compensatory PTHrP response at week 2 was lost. Damron et al.[37] interpreted these results as follows: The importance of the PTHrP axis in recovery of the growth plate after irradiation is underscored. In our study, PTHrP expression reached its lowest level at day 1 after irradiation not week 1 in contrast previous result. On the other part, recovery response was apparent at week 2 after last dose of radiation in both different models. In our study, PTHrP expression levels had poor correlation to histomorphological alterations of recovery. This discrepancy is probably related to the administration of different fractionation models. We applied a clinically relevant fractionated model unlike Damron et al.'s[4] single dose of 17.5 Gy model. Indeed, lowest level of PTHrP expression was almost on week 1 after the first fraction day in our five fractionated model. Hence, there is a possible explanation for this. The reduction in PTHrP expression probably begins with the administration of the first radiation dose, and main effect of this reduction is increased apoptosis in early period. Whereas the recovery response emerges only after radiotherapy is ended regardless of fractionation applied and is relatively independent from PTHrP activity. In a word, the roles of proliferative cytokines and growth factors in recovery response are probably only regulatory not driving.

The gene expression pattern of growth plate undergoes a dramatic change when the recovery response emerges.[6,7,36,38] Pritchard et al.[7] showed that the transcriptional shift occurs in all growth plate zones, with the sharpest alteration in reserve zone. This finding is quite important in these respects; first, stem cell-like progenitor cells are located in reserve zone and second reserve zone has a capacity for regeneration of proliferative and hypertrophic zone.[41] More importantly, differentially expressed genes were responsible for regeneration, skeletal development, bone remodeling, and ossification. This remarkable alteration implies the presence of a major transcriptional regulator for emergence of recovery response. The transcriptional factors YAP and TAZ are well known to regulate developmental, regeneration, differentiation process in various organs.[21,42] Recently, numerous studies have shown that YAP/TAZ is crucial for skeletal development and endochondral ossification.[22,23,25,26,43,44] And, besides, in a study conducted in YAP1 overexpressed transgenic mice, Deng et al.[23] demonstrated that the healing process after fracture damage is impaired due to abnormal differentiation. Considering all this knowledge, YAP and TAZ can be potent modulators for recovery response after irradiation injury. However, this hypothesis has never been examined before.

The main aim of our study was to evaluate the concordance between the change in nuclear YAP/TAZ expression pattern and histological findings of recovery response. The significant decrease in YAP/TAZ expression during the initial arrest phase, followed by a pronounced increase in YAP/TAZ expression concurrent with clonogenic proliferation, provides us with indirect evidence that YAP/TAZ may serve as a key regulator for the recovery response. The functional and structural restoration of growth plate after radiation injury depends upon the proper functioning of reparative steps.[45] Herein, optimal recovery response after radiation injury requires three essential regulations consist of the following steps: The first of this is modulation of stem cell fate, another one is cell proliferation and clonal expansion, and the last is the regulation of proper differentiation and extracellular matrix production. As stated previously, we have satisfactory evidence for the key role of YAP/TAZ in normal endochondral ossification process in these contexts as stated previously. However, when it comes to endochondral recovery upon injury, our ideas regarding YAP and TAZ appear to be only speculation, for the moment at least.

The study conducted by Rocchi et al.[27] shed light on the significant role of YAP in regeneration upon radiation. According to their results, the induction of YAP nuclear translocation with inhibition of MST1 and MST2 in salivary gland cells increased the survival fraction and proliferation of stem cells and the renewal capacity. In our model, the nuclear expression of YAP1/TAZ in the irradiated growth plate did not reach the normal level in the non-irradiated groups, despite the significant increase during recovery phase.

Also, regenerative capacity appears to be limited. The regenerative and proliferative capacity loss due to early vascularization and ossification. Inadequate YAP/TAZ activity can account for incomplete recovery response and unavoidable growth plate arrest. Having regard to the evidence of Rocchi et al.,[27] promotion of YAP or TAZ can increase the recovery capacity of the growth plate to irradiation and prevent growth arrest. Based on our own findings and considering the regenerative functions of YAP/TAZ observed in other organs including skin, liver, heart, and salivary gland,[27,42] we believe that there is a need for further investigation into the involvement of YAP and TAZ in the recovery response in growth plate after radiation injury.

Limitations of the Study

Despite we chose clinically relevant model, several limitations should be taken into consideration in interpreting the findings of this study. First, and most importantly, our evidence is indirect and are based on multiple assumptions. Although the initial results of this study are encouraging to guide further research, the available evidence are still insufficient to establish a conclusive clinical and molecular model. Another significant shortcoming is that immunohistochemistry staining is relatively subjective. We evaluated phosphorylated nuclear expression level of YAP and TAZ, their transcriptionally active forms. However, to obtain more objective results, quantitative assessments such as Western blotting and immunofluorescent staining are necessary. Additionally, YAP1 and TAZ can have zone specific and different roles. Normal distinctive zonal structure is disrupted by irradiation. Although zonal structures are not clearly observed after radiation, the expressions of YAP/TAZ may differ in the reserve zone versus non-reserve zones or in regenerative clones compared to non-regenerative cells. In further researches, region-specific assessment must be applied.

CONCLUSION

In conclusion, after the initial inhibitory effects of radiation on the growth plate, a recovery response occurs. Despite this early regenerative response, neither an aggressive catch-up growth undergoes nor the transition toward early ossification be prevented. The alteration in YAP and TAZ expression patterns provides a logical explanation for both the initial inhibitory effects and the subsequent recovery response. Insufficient activity of YAP/TAZ could potentially account for the incomplete recovery response.

Peer-review: Externally peer-reviewed.

Conflict of Interest: All authors declared no conflict of interest.

Ethics Committee Approval: The study was approved by the Gazi University Animal Experiments Local Ethics Committee (no: 20.043, date: 26/06/2020).

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REFERENCES

1. Jairam V, Roberts KB, Yu JB. Historical trends in the use of radiation therapy for pediatric cancers: 1973-2008. Int J Radiat Oncol Biol Phys 2013;85:e151–5.
2. Gawade PL, Hudson MM, Kaste SC, Neglia JP, Wasilewski-Masker K, Constine LS, et al. A systematic review of selected musculoskeletal late effects in survivors of childhood cancer. Curr Pediatr Rev 2014;10:249–62.
3. Argüelles F, Gomar F, Garcia A, Esquerdo J. Irradiation lesions of the growth plate in rabbits. J Bone Joint Surg Br 1977;59:85–8.
4. Damron TA, Mathur S, Horton JA, Strauss J, Margulies B, Grant W, et al. Temporal changes in PTHrP, Bcl-2, Bax, caspase, TGF-beta, and FGF-2 expression following growth plate irradiation with or without radioprotectant. J Histochem Cytochem 2004;52:157–67.
5. Pateder DB, Eliseev RA, O'Keefe RJ, Schwarz EM, Okunieff P, Constine LS, et al. The role of autocrine growth factors in radiation damage to the epiphyseal growth plate. Radiat Res 2001;155:847–57.
6. Damron TA, Zhang M, Pritchard MR, Middleton FA, Horton JA, Margulies BM, et al. Microarray cluster analysis of irradiated growth plate zones following laser microdissection. Int J Radiat Oncol Biol Phys 2009;74:949–56.
7. Pritchard MR, Horton JA, Keenawinna LS, Damron TA. Microarray analysis of irradiated growth plate zones following laser microdissection shows later importance of differentially expressed genes during radiorecovery. Cells Tissues Organs 2010;192:240–9.
8. Damron TA, Spadaro JA, Horton JA, Margulies BS, Strauss JA, Farnum CE. Novel radioprotectant drugs for sparing radiation-induced damage to the physis. Int J Radiat Biol 2004;80:217–28.

Turk J Oncol 2024;39(1):39–49
doi: 10.5505/tjo.2023.4213

9. Damron TA, Spadaro JA, Horton JA, Margulies BS, Strauss JA, Farnum CE. Combinations of radioprotectants spare radiation-induced damage to the physis. Clin Orthop Relat Res 2004;426:110–16.
10. Spadaro JA, Horton JA, Margulies BS, Luther J, Strauss JA, Farnum CE, et al. Radioprotectant combinations spare radiation-induced damage to the physis more than fractionation alone. Int J Radiat Biol 2005;81:759–65.
11. Damron TA, Margulies BS, Strauss JA, O'Hara K, Spadaro JA, Farnum CE. Sequential histomorphometric analysis of the growth plate following irradiation with and without radioprotection. J Bone Joint Surg Am 2003;85:1302–13.
12. Hoeben BA, Carrie C, Timmermann B, Mandeville HC, Gandola L, Dieckmann K, et al. Management of vertebral radiotherapy dose in paediatric patients with cancer: Consensus recommendations from the SIOPE radiotherapy working group. Lancet Oncol 2019;20:e155–66.
13. Margulies BS, Horton JA, Wang Y, Damron TA, Allen MJ. Effects of radiation therapy on chondrocytes *in vitro*. Calcif Tissue Int 2006;78:302–13.
14. Damron TA, Horton JA, Naqvi A, Margulies B, Strauss J, Grant W, et al. Decreased proliferation precedes growth factor changes after physeal irradiation. Clin Orthop Relat Res 2004;422:233–42.
15. Horton JA, Margulies BS, Strauss JA, Bariteau JT, Damron TA, Spadaro JA, et al. Restoration of growth plate function following radiotherapy is driven by increased proliferative and synthetic activity of expansions of chondrocytic clones. J Orthop Res 2006;24:1945–56.
16. Wang Y, Zhang M, Middleton FA, Horton JA, Pritchard M, Spadaro JA, et al. Connective tissue growth factor and insulin-like growth factor 2 show upregulation in early growth plate radiorecovery response following irradiation. Cells Tissues Organs 2007;186:192–203.
17. Damron TA, Horton JA, Pritchard MR, Stringer MT, Margulies BS, Strauss JA, et al. Histomorphometric evidence of growth plate recovery potential after fractionated radiotherapy: An *in vivo* model. Radiat Res 2008;170:284–91.
18. Meng Z, Moroishi T, Guan KL. Mechanisms of Hippo pathway regulation. Genes Dev 2016;30:1–17.
19. Fu V, Plouffe SW, Guan KL. The Hippo pathway in organ development, homeostasis, and regeneration. Curr Opin Cell Biol 2017;49:99–107.
20. Zhao B, Tumaneng K, Guan KL. The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. Nat Cell Biol 2011;13:877–83.
21. Zheng Y, Pan D. The Hippo Signaling Pathway in Development and Disease. Dev Cell 2019;50:264–82.
22. Vanyai HK, Prin F, Guillermin O, Marzook B, Boeing S, Howson A, et al. Control of skeletal morphogenesis by the Hippo-YAP/TAZ pathway. Development 2020;147:1–16.
23. Deng Y, Wu A, Li P, Li G, Qin L, Song H, et al. Yap1 regulates multiple steps of chondrocyte differentiation during skeletal development and bone repair. Cell Rep 2016;14:2224–37.
24. Goto H, Nishio M, To Y, Oishi T, Miyachi Y, Maehama T, et al. Loss of Mob1a/b in mice results in chondrodysplasia due to YAP1/TAZ-TEAD-dependent repression of SOX9. Development 2018;145:dev159244.
25. Yang B, Sun H, Song F, Yu M, Wu Y, Wang J. YAP1 negatively regulates chondrocyte differentiation partly by activating the β-catenin signaling pathway. Int J Biochem Cell Biol 2017;87:104–13.
26. Li Y, Yang S, Qin L, Yang S. TAZ is required for chondrogenesis and skeletal development. Cell Discovery 2021;7:26.
27. Rocchi C, Cinat D, Serrano Martinez P, Bruin ALJ, Baanstra M, Brouwer U, et al. The Hippo signaling pathway effector YAP promotes salivary gland regeneration after injury. Sci Signal 2021;14:eabk0599.
28. La Verde G, Artiola V, Pugliese M, La Commara M, Arrichiello C, Muto P, et al. Radiation therapy affects YAP expression and intracellular localization by modulating lamin A/C levels in breast cancer. Front Bioeng Biotechnol 2022;10:969004.
29. Zhang Y, Wang Y, Zhou D, Wang K, Wang X, Wang X, et al. Radiation-induced YAP activation confers glioma radioresistance via promoting FGF2 transcription and DNA damage repair. Oncogene 2021;40:4580–91.
30. Damron TA, Spadaro JA, Margulies B, Damron LA. Dose response of amifostine in protection of growth plate function from irradiation effects. Int J Cancer 2000;90:73–9.
31. Damron TA, Spadaro JA, Tamurian RM, Damron LA. Sparing of radiation-induced damage to the physis: Fractionation alone compared to amifostine pretreatment. Int J Radiat Oncol Biol Phys 2000;47:1067–71.
32. Pateder DB, Rosier RN, Schwarz EM, Reynolds PR, Puzas JE, D'Souza M, et al. PTHrP expression in chondrocytes, regulation by TGF-beta, and interactions between epiphyseal and growth plate chondrocytes. Exp Cell Res 2000;256:555–62.
33. Damron TA, Margulies B, Biskup D, Spadaro JA. Amifostine before fractionated irradiation protects bone growth in rats better than fractionation alone. Int J Radiat Oncol Biol Phys 2001;50:479–83.
34. Pateder DB, Sheu TJ, O'Keefe RJ, Puzas JE, Schwarz EM, Constine LS, et al. Role of pentoxifylline in preventing radiation damage to epiphyseal growth plate chondrocytes. Radiat Res 2002;157:62–8.
35. Margulies B, Morgan H, Allen M, Strauss J, Spadaro J, Damron T. Transiently increased bone density after irradiation and the radioprotectant drug amifostine in a rat model. Am J Clin Oncol 2003;26:e106–14.

36. Wang Y, Middleton F, Horton JA, Reichel L, Farnum CE, Damron TA. Microarray analysis of proliferative and hypertrophic growth plate zones identifies differentiation markers and signal pathways. Bone 2004;35:1273–93.
37. Damron TA, Horton JA, Naqvi A, Loomis RM, Margulies BS, Strauss JA, et al. Combination radioprotectors maintain proliferation better than single agents by decreasing early parathyroid hormone-related protein changes after growth plate irradiation. Radiat Res 2006;165:350–8.
38. Zhang M, Pritchard MR, Middleton FA, Horton JA, Damron TA. Microarray analysis of perichondral and reserve growth plate zones identifies differential gene expressions and signal pathways. Bone 2008;43:511–20.
39. Farquharson C, Jefferies D, Seawright E, Houston B. Regulation of chondrocyte terminal differentiation in the postembryonic growth plate: The role of the PTHrP-Indian Hedgehog Axis. Endocrinology 2001;142:4131–40.
40. Bakker B, van der Eerden BC, Koppenaal DW, Karperien M, Wit JM. Effect of X-irradiation on growth and the expression of parathyroid hormone-related peptide and Indian hedgehog in the tibial growth plate of the rat. Horm Res 2003;59:35–41.
41. Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, et al. The role of the resting zone in growth plate chondrogenesis. Endocrinology 2002;143:1851–7.
42. Moya IM, Halder G. Hippo-YAP/TAZ signalling in organ regeneration and regenerative medicine. Nat Rev Mol Cell Biol 2019;20:211–26.
43. Pan JX, Xiong L, Zhao K, Zeng P, Wang B, Tang FL, et al. YAP promotes osteogenesis and suppresses adipogenic differentiation by regulating β-catenin signaling. Bone Res 2018;6:18.
44. Zarka M, Haÿ E, Cohen-Solal M. YAP/TAZ in bone and cartilage biology. Front Cell Dev Biol 2021;9:788773.
45. Krafts KP. Tissue repair: The hidden drama. Organogenesis 2010;6:225–33.