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Apoptosis in the craniofacial tissues of irradiated growing rats

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ABSTRACT

Purpose : The purpose of this study was to investigate the apoptosis induction in tissues constituting the craniofacial region of growing rat by irradiation.

Materials and Methods : The submandibular gland, brain, articular cartilage of condylar head, and calvarium were extracted from 20-day-old rats irradiated 10 Gy. Apoptosis of each tissue was examined by DNA fragmentation and estimated quantitatively using apoptotic index on TUNEL assay. Apoptotic index of each tissue was calculated by the equation for apoptotic cells/total cells × 1,000 on the images of confocal laser scanning microscopy. Apoptotic index was analyzed statistically according to the time lapse after irradiation on the tissues.

Results : In the submandibular gland, apoptotic index was significantly increased from 6 hours after irradiation showing the highest value at 12 hours and decreased to the control level at 3 days after irradiation. In the brain, apoptotic index was abruptly reached to the maximum value at 6 hours after irradiation and decreased to the control level at 4 days after irradiation. Articular cartilage and calvarium showed no or little apoptotic signals. The results obtained by the apoptotic index accorded with that of DNA fragmentation.

Conclusion : Radiation was closely related with the apoptosis of submandibular gland and brain but, not related with the apoptosis of the articular cartilage of condylar head and calvarium. The changes induced by radiation of the hard tissues would not be explained by apoptosis. (***Korean J Oral Maxillofac Radiol 2001; 31 : 227-33***)

KEY WORDS : radiation, ionizing; DNA fragmentation; in situ nick-end labeling; microscopy, confocal

Introduction

Apoptosis is a distinct mode of cell death that is clearly different from necrosis by its characteristic morphology and biochemical features.[1,2] Morphologic characteristics of apoptosis include cytoplasmic boiling, chromatin condensation, and DNA fragmentation of nucleus.[3] Apoptosis occurs during embryogenesis, tissue remodeling, and homeostasis, as well as in pathologic conditions.[1,4] Various stimuli like heat, therapeutics, and radiation can also induce apoptosis of cells.[5-8]

There are numerous methods to measure apoptosis; loss of cell viability, DNA fragmentation, cell and nuclear morphology, cysteine protease activation, fluorescence activated cell sorting (FACS) analysis and inhibition by Bcl-2. DNA fragmentation is a simple method using the standard agarose gel stained with ethidium bromide. Recently, several authors described an in situ terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay that permits detection of apoptosis on paraffin-embedded tissue sections.[9,10]

For the treatment of head and neck cancers, radiotherapy is often employed to improve local control. It is usually unavoidable to include other healthy organs in head and neck area. Radiation can lead dysfunction and impairment of organs, retardation of growth, damage of cells, and so on.[11-13] It was made efforts to try to assess the relationship between apoptosis and dysfunction of the organ,[14] and radiosensitivity.[15] There were many studies on radiation induced apoptosis,[16-18] and it was known that radiation induced apoptosis was varied by the type of cell and irradiation.[16]

Radiosensitivity is varied according to the irradiated tissues, and apoptosis can also be involved variously as a contributing factor to the sensitivity of target-cell and hence to early reactions in tissues after irradiation. It is still unclear whether, or how, the mode of cell death determines the radiosensitivity.[19]

In this study, we evaluated the apoptosis in four tissues constituting the craniofacial area of growing rats according to the time lapse after irradiation. The tissues were the submandibular gland, brain, articular cartilage of condylar head and calvarium. Apoptosis was assessed by DNA fragmentation and quantitative evaluation was performed by apoptotic index

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(apoptotic cells/total cells × 1000) on the image stained by TUNEL assay. The images were acquired by confocal laser scanning microscopy using argon laser. Apoptotic index was analyzed statistically according to the time lapse after irradiation on the tissues.

Materials and Methods

1. Experimental animals and irradiation

Twenty-day-old 48 male Sprague-Dawley rats were used for this study. Animals were housed in temperature and humidity-controlled conditions. The animals were kept on a high 12 hour light cycle with food and water.

All animals except six animals for control group were irradiated with a single dose of 10 Gy gamma irradiation delivered by MK 1 irradiator (Shepherd & Associates, USA) using Cs-137 beam with a dose rate of 237 cGy/min. The irradiation were performed limitedly on the craniofacial area of animals.

The rats were classified by eight groups according to the sacrifice time; control, 6, 12 hours, 1, 2, 3, 4, and 5 days after irradiation. Each group was constituted with 6 experimental animals. The submandibular gland, brain, articular cartilage of condylar head, and calvarium were extracted from all animals. The tissues from three animals of each group were used for DNA fragmentation, and the others for TUNEL assay.

2. DNA fragmentation analysis

For DNA fragmentation, extracted specimens were dissected out under aseptic conditions. The articular cartilages of condylar heads were grinded with knife for analysis. The samples were placed in digestion buffer (50 mM Tris-Cl pH 8.0, 100 mM EDTA, 100 mM NaCl, 5% Triton X-100) with 0.1 mg/ml proteinase K at 55°C till the mixture became clear. The DNA was extracted by phenol/chloroform/isoamyl alcohol (25 : 24 : 1), and centrifuged at 2,000 g for 5 minutes. The sample was re-extracted with chlorofom/isoamyl alcohol (24 : 1) at 2,000 g for 5 minutes. The supernatant was transferred to a fresh tube and, added by 2 volumes of 100% ethanol with one -fifth volume of ammonium acetate. The DNA was precipitated overnight in −20°C. It was centrifuged for 5 minutes, 4°C at 12,000g. DNA pellets were recovered by centrifugation (12, 000g for 1 minute), washed with 70% ethanol, air-dried, and resuspended in 100 μl of TE buffer. The sample added by RNase was incubated for 30 minutes at 37°C. DNA (10 μg) was quantified with spectrophotometer, and electrophoretically separated on a 2% agarose gel containing 1 μg/ml ethidium bromide for 50 minutes at 90 V, 10 mA. Pictures were taken by UV transilluminator.

3. TUNEL assay

The specimens obtained from all the tissues were fixed 10% neutral buffered formalin for 1 week. The articular cartilage was decalcified with pH 7.4, 10% EDTA solution for 1 week. They were embedded in paraffin. Three sections were obtained from the paraffin block with 5 μm thickness. Before staining, the sections were routinely deparaffinized through xylene and graded alcohol.

Apoptosis was examined with TUNEL assay using ApopTag Kit (Oncor, Gaithersburg, USA). Referring the manual, the staining was performed. In briefly, after proteinase digestion for 15 minutes, the specimen was washed in 4 changes of distilled water for 2 minutes each wash. It was treated by working strength TdT (terminal deoxynucleotidyl transfrease) enzyme, applied with working strength anti-digoxigenin-fluorescein. Propidium iodide was used for counterstaining.

4. Apoptotic index evaluation

Three areas from each sample were selected randomly on the image of confocal laser scanning microscopy (CLSM, BIO

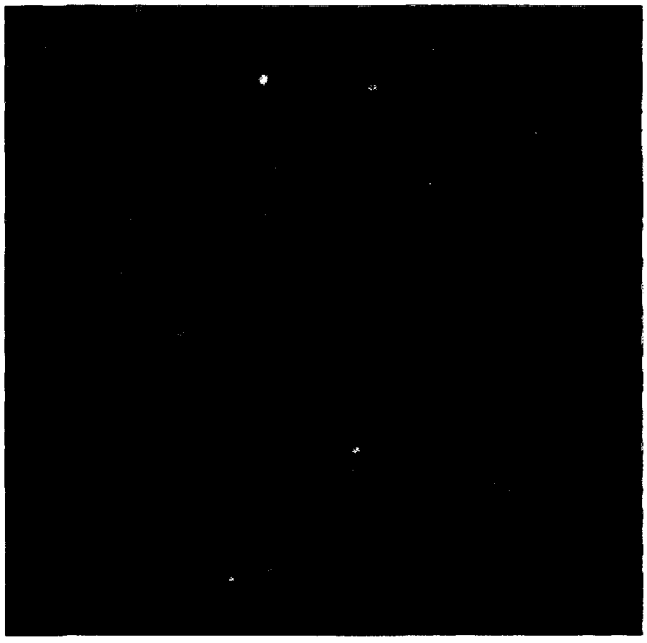


Fig. 1. Confocal image of the submandibular gland (12 hours after irradiation, × 200). There were a few yellow signals for the apoptotic cells.

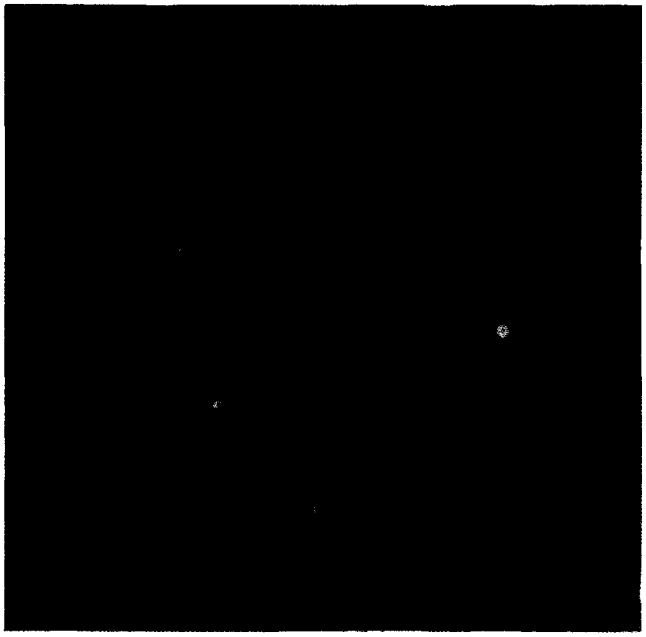


Fig. 2. Confocal image of the brain (6 hours after irradiation, × 200). There were a few yellow signals for the apoptotic cells.

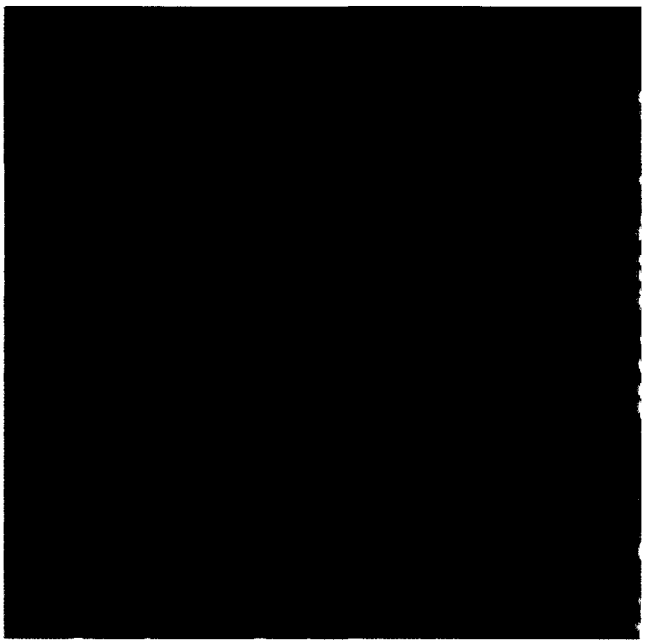


Fig. 3. Confocal image of the articular cartilage of condylar head (12 hours after irradiation, × 200). There was no apoptotic signals.

-RAD, MRC 1024). Argon laser of CLSM was used with 15 mA, excited on 488 nm. The number of apoptotic signals (green) and total cells (red) were counted on the each image (× 200) (Figs. 1-4). Apoptotic index was calculated by the equation for apoptosis positive cells/total cells × 1,000 on each

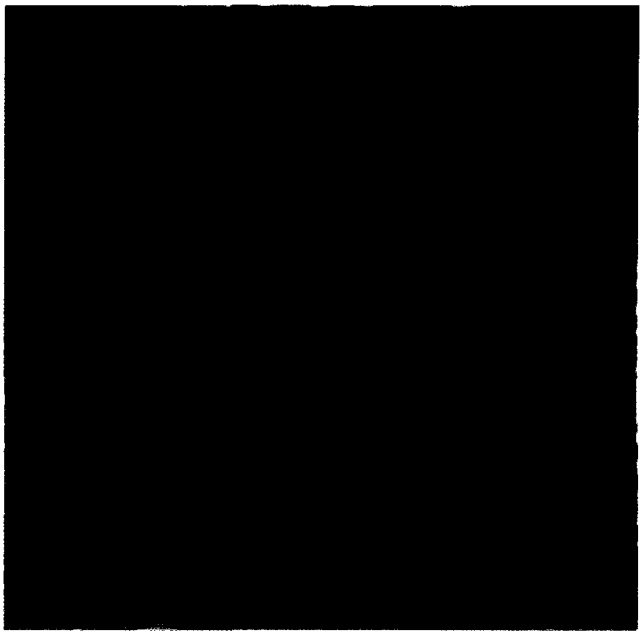


Fig. 4. Confocal image of the calvarium (12 hours after irradiation, × 200). There was no apoptotic signals.

image and evaluated statistically as the time lapse according to the tissues. T-test was applied to analyze statistically.

Results

1. DNA fragmentation analysis

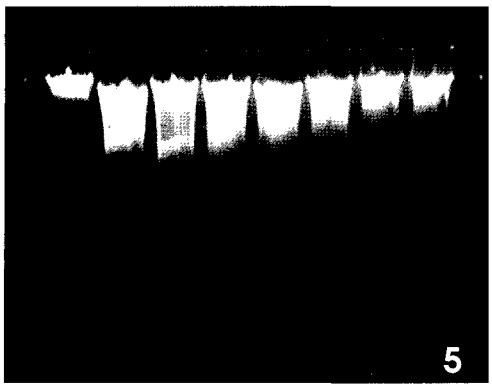
In the submandibular gland, DNA fragmentation was observed in all irradiated groups, while not observed in control group. The amount of DNA fragmentation was significant within 1 day after irradiation, and decreased with the time lapse, to the extent of control group after 2 days (Fig. 5).

In the brain, it showed similar pattern of the submandibular gland. The amount of DNA fragmentation was significant within 3 days after irradiation. It had a tendency to decrease gradually with the time lapse. DNA fragmentation from 4 days after irradiation showed little difference compared to control group (Fig. 6).

In the articular cartilage of condylar head and the calvarium, no DNA fragmentation was observed in the all groups after irradiation as well as control group (Figs. 7, 8).

2. Apoptotic index evaluation

In the submandibular gland, control group showed little apoptosis. Apoptotic indices of the irradiated groups were increased gradually with the time lapse showing the highest at



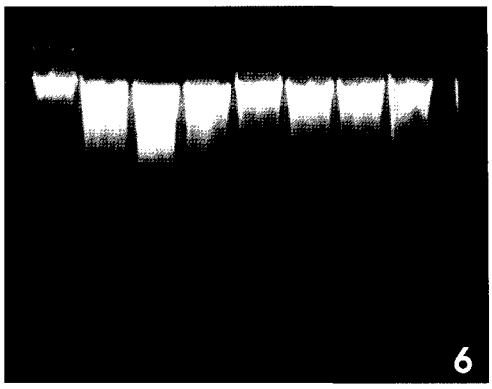
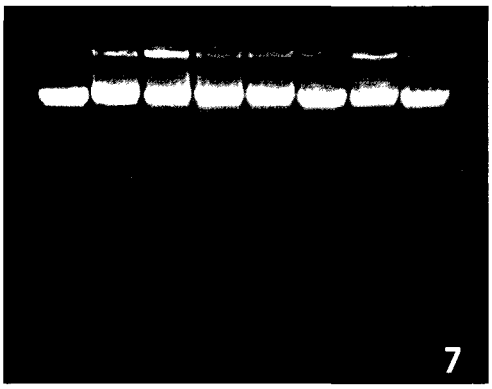


Fig. 5, DNA fragmentation analysis of the submandibular gland. DNA fragmentation was significant within 1 day after irradiation and decreased gradually with the time lapse.

Fig. 6. DNA fragmentation analysis of the brain. DNA fragmentation was significant within 3 days after irradiation and decreased gradually with the time lapse.



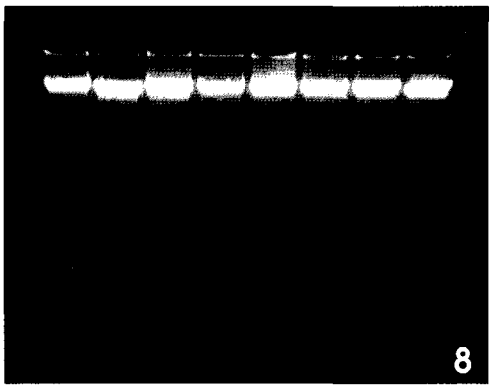


Fig. 7. DNA fragmentation analysis of the articular cartilage of condylar head. No DNA fragmentation was observed in all groups after irradiation as well as control group.

Fig. 8. DNA fragmentation analysis of the calvarium. No DNA fragmentation was observed in all groups after irradiation as well as control group.

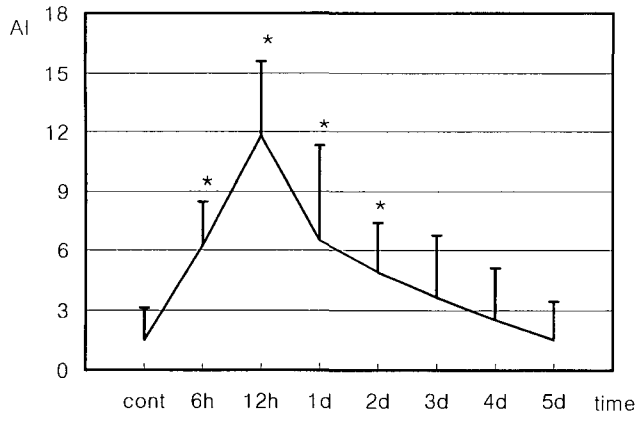


Fig. 9. Apoptotic index of the submandibular gland according to the time lapse after irradiation. *: statistically significant difference compared with control group.

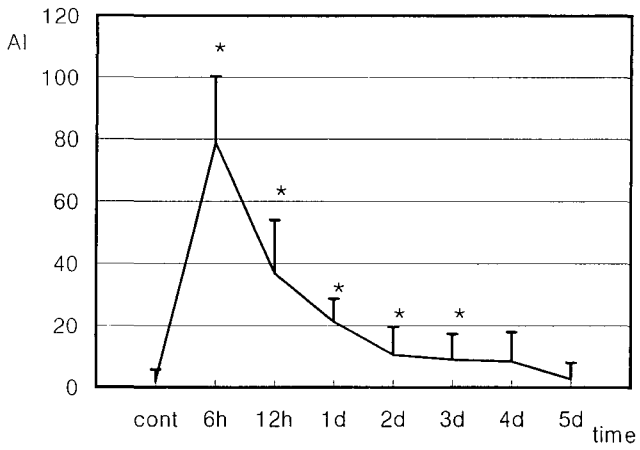


Fig. 10. Apoptotic index of the brain according to the time lapse after irradiation. *: statistically significant difference compared with control group.

12 hours after irradiation, and afterward decreased gradually. Apoptotic index showed statistically significant difference between control and 6 hours irradiation group, 6 hours and 12 hours, and 12 hours and 1 day. There was statistically significant difference in 6 hours, 12 hours, 1 day, 2 days group compared with control group (Fig. 9).

In the brain, control group showed little apoptosis like in the submandibular gland. Apoptotic index showed the highest value at 6 hours after irradiation, and decreased gradually with the time lapse. There was statistically significant difference between control and 6 hours, 6 hours and 12 hours, 12 hours and 1 day, and 1 day and 2 days. The groups of 6 hours, 12 hours, 1 day, 2 days, and 3 days after irradiation showed statistically significant difference compared with control group

(Fig. 10). In the articular cartilage of condylar head and the calvarium, there were no or little apoptotic signals (Figs. 3, 4) in all groups after irradiation as well as control group. The results of apoptotic index evaluation accorded with that of DNA fragmentation analysis for all the tissues.

Discussion

Radiation causes impairment of the tissues, change of cell size, and loss of cells,[20] and the extent of injury is affected by various factors. The effect of irradiation is varied according to the characteristics of tissues,[21] and radiation induced apoptosis is also to them.[22] In present study, irradiation was performed by a single dose of 10 Gy limitedly in craniofacial region of the animals. Radiation field limitation except craniofacial region could be performed with lead block specially designed for the irradiator. The dose was determined considering $LD_{50/30}$ of rats.[23] It is generally known that radiation induces dysfunction of tissue. Radiation treated patients due to cancer in head and neck area showed xerostomia by impairment of acinar cell, cell loss, and morphological changes.[24] Recently, some authors reported that the cell death by irradiation was due to programmed cell death, or apoptosis.[25-27] Apoptosis was thought to be an origin of dysfunction of tissues,[28,29] but Paardekooper et al.[16] studied that the malfunction of salivary gland by impairment due to radiation could not be explained by apoptosis, alternative mechanisms were proposed. Apoptosis might be closely correlated with radiosensitivity, 30 but it was controversial and many factors had to be considered.[26,27]

Apoptosis has been assessed by examination of morphological changes with light and electron microscopy. Recently, various methods have been introduced,[31] and some methods were designed for quantitative analysis. Apoptosis could be detected simply by DNA fragmentation,[32] and recently, new DNA fragmentation using digital imaging could analyze apoptosis qualitatively and quantitatively.[33] TUNEL assay was also useful for detection of apoptosis,[34-37] which identifies 3'-OH ends of DNA-strand breaks, has been widely used as a marker of DNA damage or repair. The method designed for this study has been shown to be the most sensitive method for quantitative evaluation of apoptosis available at present.[38] The examination of apoptosis has been usually performed by light microscopy or fluorescent microscopy.[39-41] Confocal laser scanning microscopy, however, was superior to, more exact tool than others, so more reliable data could be acquired. Apoptotic index could be calculated by the equation for apoptosis positive cells/total cells $\times$ 1,000 on the microscopic images that had been stained by conventional H & E stain or TUNEL assay. The value of apoptotic index from each tissues were closely correlated with the results from DNA fragmentation.[42-44]

In this study, apoptosis was detected by DNA fragmentation and apoptotic index was calculated on the images of confocal laser scanning microscopy. In the submandibular gland, DNA fragmentation was increased gradually to 12 hours after irradiation, and then decreased gradually with the time lapse. Apoptotic index showed statistically significant difference between control group and 6 hours group, 6 hours group and 12 hours group, and 12 hours group and 1 day group. Apoptotic index of 3 days, 4 days, and 5 days after irradiation showed no statistically significant difference comparing with that of control group, or the recovery of the impairment by irradiation was done after 3 days.

In the brain, DNA fragmentation was significant within 3 days after irradiation. From 12 hours after irradiation, It had a tendency to decrease gradually with the time lapse. Apoptotic index of all irradiated group showed higher than control group, but there was no statistically significant difference between 4 days and 5 days group after irradiation and control group. Brain showed the highest apoptotic index within 6 hours after irradiation, and then decreased gradually to control level from 4 days. It was similar results obtained in the previous study that the number of pyknotic cells were the highest between 4 and 8 hours after gamma irradiation.[18] The results of DNA fragmentation showed closely related to those of apoptotic index evaluation.

The difference of apoptotic indices was not analysed statistically between submandibular gland and brain because the direct comparison had not performed while it was expected that there would be the significant difference of apoptotic index between them. For the direct comparison between the tissues, it might be required the comparison in the same experimental condition and on one agarose gel and the evaluation of equal number of cells for apoptotic index evaluation.

Articular cartilage of condylar head and calvarium, there was no DNA fragmentation. Also, no or little apoptotic signal was observed in all samples on the images of confocal laser scanning microscopy. Radiation used in this study could not induce apoptosis for them. In the previous study on apoptosis in the development of the temporomandibular joint, there was no apoptosis in gestation period and postnatal period except postnatal 1 day.[4] It was accorded with control group of articular cartilage in present study although 20-day-old rats were used in this study, moreover radiation did not induce apoptosis of the articular cartilage of condylar head. Irradiated mouse

showed, however, decrease of the thickness of all zones and the number of the cells of articular cartilage.[20,45] The decrease of the number of cells might not be due to apoptosis, but the retardation of cell proliferation. In the previous study on apoptosis of calvarium, it was demonstrated that apoptosis played an important role at the site of sutural growth.[46-49] It was also known that apoptosis could be occurred during normal development and bone remodeling.[50] But in this study, none or little apoptosis was shown because apoptotic cells might be phagocytosed rapidly and irradiation affect minimally apoptosis of calvarium in growing rats.

It was probably that there might be some differences in the induction of apoptosis between hard tissues for calvarium and articular cartilage and soft tissue for submandibular gland and brain. In the hard tissues, no apoptosis showed in all groups, but it was known that radiation could affect the destruction and dysfunction of the tissues. It must be studied what is the origin of the destruction of the hard tissues.

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