doi:10.1111/j.1365-2591.2007.01294.x

The effect of head-fractioned teletherapy on pulp tissue

F. V. Vier-Pelisser[1], M. A. Z. Figueiredo[1], K. Cherubini[1], A. Braga Filho[2] & J. A. P. Figueiredo[3]

[1]Department of Oral Medicine; [2]Radiotherapy Unit of Sao Lucas Hospital, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre Brazil; and [3]Unit of Endodontology, Eastman Dental Institute, University College London, London, UK

Abstract

Vier-Pelisser FV, Figueiredo MAZ, Cherubini K, Braga Filho A, Figueiredo JAP. The effect of head-fractioned teletherapy on pulp tissue. *International Endodontic Journal*, **40**, 859–865, 2007.

Aim To evaluate the early and delayed effects of fractioned teletherapy (radiotherapy) on the dental pulps of rats using Co^{60}.

Methodology In group 1 – rats ($n = 15$) were subjected to fractioned teletherapy by 30 daily sessions fractioned in doses of 200 cGy day^{-1}, totaling 60 Gy and the rats were killed immediately after the final dose of irradiation; group 2 – same protocol but killed 30 days following the final irradiation dose; groups 3 ($n = 7$) and 4 ($n = 8$) – formed controls without irradiation. Following perfusion, the left mandible of each rat was dissected and processed for histopathology. Serial sections (5 μm) were obtained and stained with HE or picrosirius. Observations were recorded for the coronal pulp tissue. A blinded observer evaluated HE sections using pre-defined indices of inflammation, nuclear alterations and extracellular matrix (ECM) hyalinization. Images of sections stained with picrosirius were converted to black and white for analysis by IMAGE-PRO PLUS; areas in black (collagen) were measured as percentage area. The pulps of mandibular incisors of the specimens prepared for transmission electron microscopy (TEM) were subjected to descriptive analysis. Magnifications of 6300 and 10000× were used to observe 10 pulp fibroblasts from each group.

Results No inflammatory reactions or modification of the ECM status were found ($P = 0.428$) in any specimens. The collagen content also displayed no significant changes ($P = 0.067$) as a result of treatment. Groups 1 and 2 displayed significantly more nuclear alterations than the control groups ($P < 0.05$). The bubble-like aspect was more pronounced in group 1, and the bubbles looked smaller in group 2. The ECM showed no differences in the hyalinization status and there were no differences in the collagen area within the pulps. Under TEM, the pulp fibroblasts in group 1 displayed nuclear alterations that resembled circular, oval or elongated perforations; perforations also appeared in the cytoplasm.

Conclusion Fractioned teletherapy is capable of producing nuclear alterations in the dental pulp tissue of rats.

Keywords: dental pulp, head, microscopy, teletherapy.

Received 14 December 2006; accepted 11 April 2007

Introduction

Radiotherapy is a therapeutic treatment for head and neck cancer treatment. It utilizes ionizing radiation to destroy neoplastic cells and thus reduces or eliminates the malignant lesion. Although it has therapeutic effects, there are complications specific to head and neck radiotherapy such as mucositis, candidiasis, xerostomia, reduced mouth opening and loss of taste (De Moor 2000) that are potentially associated with increases in the incidence of carious lesions in teeth. In a Dutch population, Nesse *et al.* (2006) reported that the prevalence of obstructive apnoea in patients subjected to head and neck cancer treatment was at least

Correspondence: Jose Antonio Poli de Figueiredo, Unit of Endodontology, Eastman Dental Institute, University College London, 256 Gray's Inn Road, London WC1X 8LD, UK (Tel.: +44 20 7915 2362; fax: +44 20 7915 2371; e-mail: j.figueiredo@eastman.ucl.ac.uk).

27 times higher following radiotherapy alone or combined with surgery.

When orthovoltage therapy was employed, there was extensive damage to pulp tissues, such as circulatory disturbances, hyaline degeneration and necrosis (Kalnis 1954). Meyer *et al.* (1962) reported complete necrosis of odontoblasts and fibroblasts with haemorrhage and inflammatory cells infiltration.

The use of Cobalt-60 (^{60}Co) instead of orthovoltage resulted in different features (Hutton *et al.* 1974, Nickens *et al.* 1977, Matson *et al.* 1978, Fawzi *et al.* 1985), with no notable differences between irradiated pulps and the control group. However, Anneroth *et al.* (1985) reported metaplasic alterations, such as fibrosis and hyaline degeneration, in head and neck irradiated patients.

The contradictory and scarce findings in the literature lead to the need for more detailed investigation of the effect of ionizing radiation on dental pulp tissue. Any disturbance in the pulp tissue leading to infection may pre-dispose to osteoradionecrosis, which is the most serious complication of head and neck radiotherapy (De Moor 2000).

The aim of this study was to evaluate the immediate and delayed effect of fractioned teletherapy by Co^{60} on pulp tissue in rats.

Materials and methods

This research was initiated following approval by the Scientific and Ethics Committee of the Dental School of PUCRS, Brazil (protocol 0106/03), and was in accordance to international regulations regarding animal studies.

Forty-five male albino rats, *Rattus norvergicus*, 80 days old, weighing between 220 and 290 g, were divided into four groups:

1. Group 1 – 15 rats submitted to 60 Gy, being killed immediately following the final radiotherapy session.

2. Group 2 – Same as group 1 (n = 15), but killed 30 days following the final radiotherapy session.

3. Group 3 (control 1) – Seven rats not submitted to radiotherapy, but killed together with experimental group 1.

4. Group 4 (control 2) – Eight rats not submitted to radiotherapy, but killed together with experimental group 2.

The head part of the rats of experimental groups 1 and 2 were subjected to daily radiotherapy sessions, for 30 days (Sagowski *et al.* 2004), at the Radiotherapy Unit of Sao Lucas Hospital, PUCRS, Brazil. A Co^{60} teletherapy machine (Philips XK 5101, Eindhoven, the Netherlands; Co^{60} font: January 1979, Ottawa, Canada) with an energy output of 1.25 MeV was used. The total radiation dose was 60 Gy, fractioned in daily doses of 200 cGy day^{-1}.

The rats were placed in a plastic container within a wooden box with openings to allow head placement at a distance between the beam and the skin surface of the rats of 60 cm and an area of irradiation field equivalent to 20 × 20 cm.

After killing, two rats of each experimental group and one rat of each control group were perfused with 2% glutaraldehyde. The other rats were subjected to perfusion with 4% paraformaldehyde. The left hemimandible was dissected and a fragment containing the three molar teeth as well as the middle third of mandibular incisors was obtained. For the specimens fixed with 2% glutaraldehyde, a cut perpendicular to the long axis of the molar roots was made to subdivide the sample into two, one containing the three molar teeth and the other containing the middle third of the mandibular incisors, which was subsequently prepared for transmission electron microscopy (TEM; Fig. 1). The samples were decalcified with 17% EDTA (pH 7.0) (Farmacia Calendula, Porto Alegre, Brazil). Serial sections of 5 μm thickness were stained with HE and picrosirius consecutively. Picrosirius is a technique used to study specifically collagen content, staining this structure in red. The elongated dye molecules are attached to the collagen fibre in such a way that their long axes are parallel. This parallel relationship between dye and collagen results in an enhanced birefringency (Junqueira *et al.* 1979). Three serial sections of 5 μm were obtained for each of the stains used.

The coronal pulp of the mandibular molar that displayed the largest area of tissue was chosen for examination, in duplicates, by one observer, blinded about the group under analysis. A light microscope (Olympus Optical Co. Ltd, model BX 41TF and

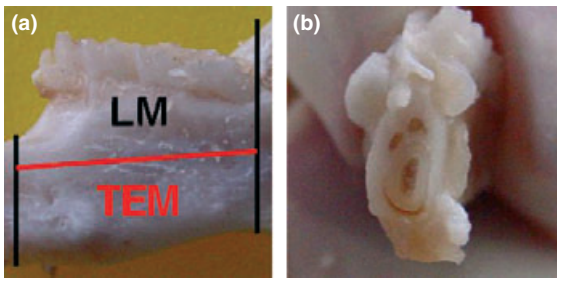

Figure 1 (a) The hemi-mandible and the area of section for LM and TEM. (b) An *in situ* view of the area of the lower incisor which the pulp fragments were obtained for TEM.

U-MDOB3, Tokyo, Japan) was used in magnifications of 40, 100 and 400×, considering the following:

- Inflammatory process
  1. *Absent* – loose connective tissue showing normal cellular content and extracellular matrix (ECM);
  2. *Present* – vascular alterations (hyperaemia and plasma exudation), neutrophil and/or lymphocyte and plasma cell infiltrates.
- Nuclear alterations
  1. *Absent* – nuclei exhibiting homogeneous staining;
  2. *Present in some cells* – altered nuclei, with weakly stained areas, resembling 'bubbles' in some cells, regardless of the cell type, but not in the majority;
  3. *Present in the majority of cells* – most pulp cells displaying the above-mentioned alterations.
- Matrix hyalinization:
  1. *Absent*– absence of hyalinized areas;
  2. *Focal* – hyalinization represented as densely stained areas, dispersed in the tissue;
  3. *Extensive* – hyalinized areas occupying most of the field under analysis.

Images of pulp tissue from the sections stained with picrosirius were captured at 200× magnification with an optical microscope (Olympus AX 70, New York, NY, USA) coupled with a digital imaging system (Olympus U-PMTVC, Salt Lake, UT, USA) and processed by the IMAGE-PRO PLUS Program (Adobe Inc., San José, CA, USA). From each image, three rectangular areas of variable size were selected and measured in μm^2 (Fig. 2). They were then inserted into the ADOBE PHOTOSHOP 7.0 software (Adobe Inc., San José, CA, USA). The colours were changed to white, except for the red areas, which contained the collagen. The second step was to remove the pale red areas, which under picrosirius staining are nonspecific material, in such a way that only vivid red areas representing collagen would be maintained. These areas were converted into black by selecting the white colour with a tolerance of 32 levels in the software. This sequence was repeated in triplicate; therefore, from each pulp image, three black and white copies were produced. The software IMAGE-PRO PLUS could then quantify the black/white ratio, the collagen content being black. The results of the area occupied by the collagen were expressed in %. For each specimen, an average of the findings was obtained (Fig. 3).

The osseous tissue of the molar fragment was sectioned with an osteotome (Edlo, Canoas, Brazil), the middle third of the mandibular was sectioned and fragments of approximately 1 mm^2 were obtained with a surgical blade and prepared for TEM.

Electron micrographs of 10 fibroblasts were obtained for each group, each in two magnifications (6300× and 10000×), using the electron microscope (Philips EM208S, Eindhoven, the Netherlands) operating at

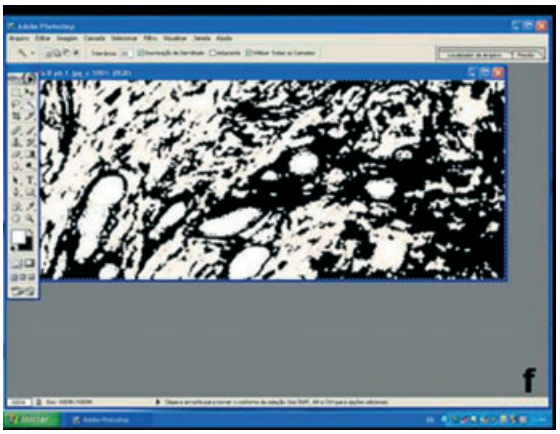

Figure 3 The final black and white image, following a series of colour substitution steps. The area corresponding to the collagen content being black.

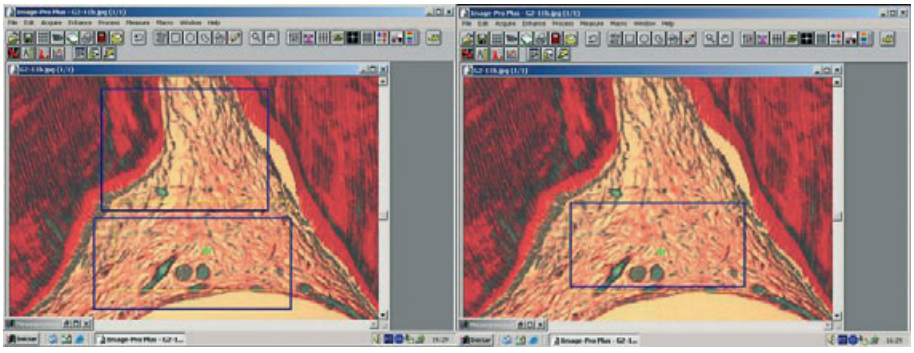

Figure 2 View of the images obtained with the sections stained with picrosirius where three distinct rectangular areas were delimitated for analysis.

80 kV. When the negatives were dried, they were scanned (Scanner Epson Perfection 3170 Photo, Model J161A, Nagano, Ken, Japan) at 600 dpi resolution.

An observer evaluated the images, particularly, observing the nuclear alterations as observed in the HE light microscopy sections.

The Kappa coefficient test was used to evaluate intra-examiner agreement, and the Kruskal–Wallis nonparametric test complemented by the multiple comparisons test, at a level of 5%, was used to evaluate the differences amongst the groups. Analysis of variance (ANOVA) was used to verify the differences in percentage of collagen content amongst the groups. The software used for the statistical analysis was SPSS 8.0 (SPSS Inc., Chicago, IL, USA).

Results

There was no inflammatory reaction in any group. The intra-examiner agreement between the first and the second observations in terms of nuclear alteration and matrix hyalinization gave a Kappa coefficient of 0.769 and 0.744, respectively.

The Kruskal–Wallis nonparametric test demonstrated no significant differences ($P = 0.428$) regarding matrix hyalinization (Tables 1 and 2). The ANOVA test confirmed there were no significant differences amongst the groups studied with regard to the average collagen content ($P = 0.067$) (Table 3). However, the average corresponding to group 2 was numerically superior (Figs 4 and 5).

The Kruskal–Wallis nonparametric test complemented by the multiple comparisons test, at a level of 5%, revealed no statistically significant differences between the test groups 1 and 2 for nuclear alteration. However, both test groups demonstrated higher levels of nuclear alterations when compared with control groups 3 and 4 (Table 4).

Table 1 ECM hyalinization levels of rats' molar coronal pulps stained with HE, for experimental and control groups

	Group			
ECM hyalinization	Group 1	Group 2	Group 3	Group 4
Absent	–	–	–	–
Focal	8	4	5	4
Extense	6	6	2	1
Total	14[a]	10[a]	07	05[a]
Average rank	18.71	21.80	16.14	14.60

Kruskal–Wallis nonparametric test: $P = 0.428$.
[a]One, four and three specimens, respectively, were lost during histology of groups 1, 2 and 4.

Table 2 Average collagen content (%) of rats' molar coronal pulps stained with picrosirius, for experimental and control groups

		Collagen content			
Group	*N*	Average (%)	SD	Minimum	Maximum
Group 1	13[a]	28.33	12.36	14.50	51.15
Group 2	9[a]	47.23	19.45	14.21	79.90
Group 3	7	35.47	9.74	24.83	49.41
Group 4	6[a]	39.09	21.14	15.49	70.84

[a]Two, four and two specimens, respectively, were lost during histology of groups 1, 2 and 4.

Table 3 ANOVA of the collagen content in rats' molar coronal pulps stained with picrosirius

Causes of variance	Degrees of liberty	Square sum	*F*-value	*P*-value
Group	3	1952.40	2.63	0.067
Experimental error	31	7663.13		
Corrected total	34	9615.53		

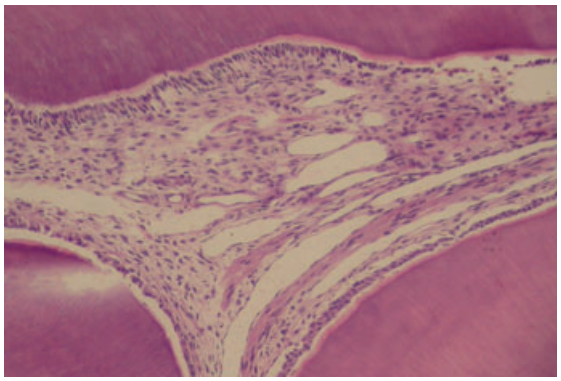

Figure 4 Light microscopy of coronal pulp of group 1 – intense hyalinization can be observed in this specimen (HE-40×).

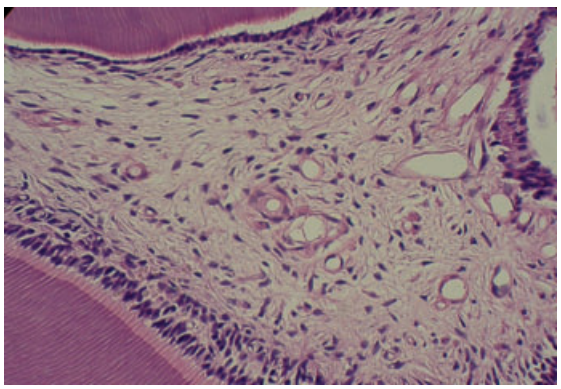

Figure 5 Light microscopy of coronal pulp of group 1 – focal areas of hyalinization can be seen (HE-40×).

Table 4 Presence and degree of nuclear alteration in rats' molar coronal pulps stained with HE

	Group			
Nuclear alteration	Group 1	Group 2	Group 3	Group 4
Absent	6	–	7	5
Present in some cells	1	7	–	–
Present in the majority of cells	7	3	–	–
Total	14[a]	10[a]	7	5[a]
Average rank	21.43[b]	25.20[b]	9.5[c]	9.5[c]

[a]One, four and three specimens, respectively, were lost during histology of groups 1, 2 and 4.
[b,c]Average ranks followed by distinct letters are significantly different at a significance level of 5%.

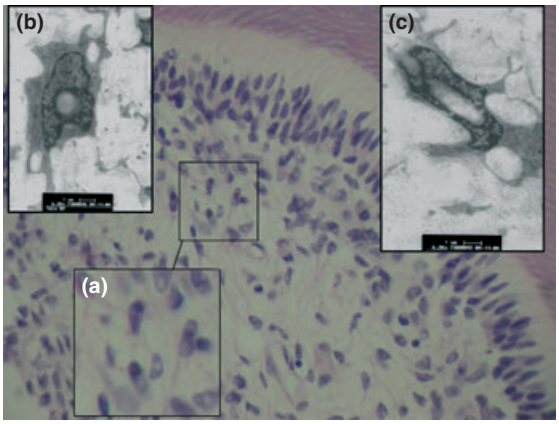

Figure 6 Coronal pulp of rats from group 1. (a) – The magnified region reveals the presence of expressive nuclear alterations in pulp fibroblasts, with bubble-like aspects (HE-400×). (b) and (c) – TEM showing nuclear and cytoplasm alterations resembling perforations ((b)-6300×; (c)-10000×).

The nuclei of some pulp cells of the irradiated rats had alterations that resembled vacuoles or bubbles, or sometimes a spherical weakly stained area, being present both in fibroblasts and odontoblasts of the molar coronal pulp. Although not statistically significant, such alterations were more obvious in rats of group 1 than group 2. The alterations in rats of group 1 were extensive, and in the majority of the cases, unique (Fig. 6). The group 2 rats displayed multiple discrete alterations with minute vacuoles or bubbles within the cell nucleus (Fig. 7a,b). Some altered cells had a higher nuclear volume than normal cells.

The nuclei of the fibroblasts and odontoblasts of control groups 3 and 4 did not exhibit any type of alteration (Fig. 8).

The TEM analysis revealed that some fibroblasts of the rats of group 1 displayed alterations both in the

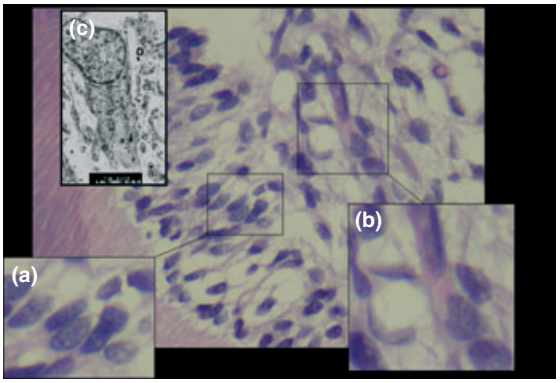

Figure 7 Coronal pulp of rats from group 2. (a) and (b) – The magnified regions (HE-400×) reveal the presence of less pronounced nuclear alterations, resembling very small bubbles; (c) – TEM (6300×) showing fibroblast with no alterations.

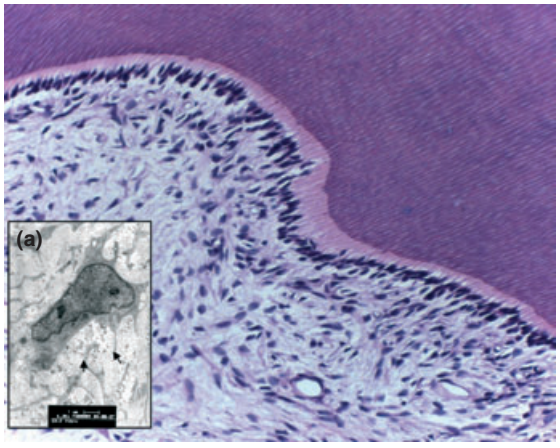

Figure 8 Coronal pulp of rats from group 4. Note the absence of nuclear alteration; some nuclei are pale but no bubble-like aspect was seen (HE-400×). (a) – TEM (6300×) shows no nuclear and cytoplasm alterations. Note the presence of transversal collagen fibre bundles (arrows).

nucleus and the cytoplasm. These alterations resembled perforations of varied sizes, being circular (Fig. 6b), ovoided or elongated (Fig. 6c). No such alterations were found in cells of groups 2 (Fig. 7c) and 3 (Fig. 8a). In all cells of group 1, the chromatin was more condensed at the periphery of the nucleus.

The findings in TEM sections of some pulp fibroblasts were similar to those seen in fibroblasts and odontoblasts of HE stained sections from groups 1 and 2 when examined using light microscopy.

Collagen fibre bundles could only be seen at the periphery of some fibroblasts of the control group (Fig. 8a).

Discussion

The aim of this study was to evaluate the immediate and delayed effects of Co^{60}-fractioned teletherapy on dental pulp tissue of rats, in order to obtain information that could be extrapolated to clinical practice and understand what could be expected to happen to the dental pulps when patients are submitted to head and neck radiotherapy. Co^{60}-fractioned teletherapy is the treatment of choice for head and neck cancer when radiotherapy is indicated. The study in animals using fractioned doses is sometimes difficult for the equipments that are generally available in hospitals, for human patients. The use of rats allowed comparison amongst groups and the influence of time (30 days) over pulp tissue response.

Hutton *et al.* (1974) and Nickens *et al.* (1977) did not find, under light microscopy, alterations in irradiated monkeys subjected to 70 and 76 Gy of Co^{60}, respectively, at experimental periods similar to the present study. However, their samples comprised of only two and one monkey, respectively, and the magnifications used were relatively modest at 80× and 35×. Therefore, some tissue alterations could have been missed. The absence of an inflammatory reaction reported here is in accordance with the two previous studies.

A large number of pulp fibroblasts and odontoblasts displayed nuclear alterations. Mettler & Upton (1995) have also reported nuclear alterations in irradiated cells. In the present study, the irradiated cells presented chromatin grouping in such a way that part of the nuclear content was lacking this element, resembling intra-nuclear weakly stained areas, vacuoles or bubbles when examined through optical microscopy. This could well be oedema, so intense as to increase the nuclear volume, a feature also observed.

The alterations found in group 1 were more obvious than those in group 2. Thirty days following irradiation, the alterations were more discrete, as if the weakly stained areas or vacuoles were fragmented, producing an image resembling minute bubbles along the nuclear surface. It could be speculated that a regrouping of DNA was occurring, conferring this aspect to the nucleus. From this study, it became evident that pulp cells are modified following radiotherapy. However, this could be a transitory event, as changes in pulp tissue within group 2 were less obvious compared with group 1.

The use of TEM in this study allowed a comparison of the findings with the light microscopy sections. It was possible to observe nuclear as well as cytoplasmic alterations, the cells being either perforated or subjected to intracellular microexplosions. It could be speculated that the X-ray beam may have gone through or collided with cells at different angles, resulting in the various features reported. These could also be attributed to variations in the plane of the sections.

The chromatin condensation at the periphery of the nucleus, with the disappearance of the borders of this structure, was noted in some fibroblasts of group 1. Not only the nucleus of the cells was affected in group 1, as seen under light microscopy, but the cytoplasm as well. There was a contradictory finding in group 2, for it did not show, under TEM, the nuclear alterations found under light microscopy. The differences could reflect the site of observation, which was the middle third of the mandibular incisors; as these teeth are constantly growing, the cells of this area could have been replaced in 30 days. In addition, pulp cellular turnover could be sufficient to recover the cellular structures or to substitute the dead cells.

Matrix hyalinization did not occur in rats of group 2 as would be expected with time. Mathes & Alexander (1996) reported both a rise in fibrosis and diminished vasculature as delayed effects of irradiation. Possibly, the 30-day period of observation in the present study was not enough to denote such variations.

Qualitative and quantitative approaches were used in this study to verify matrix hyalinization and collagen content. The use of picrosirius and the adjunct software manipulation of the images to reveal more accurately the area where the collagen was present may be useful in future studies.

The information obtained from rat models should be interpreted with caution. Although the dose could be considered high for such small specimens, humans have pulp alterations because of caries, aging, trauma, attrition, abrasion, erosion, restorative preparations, etc., which can diminish its reparative potential.

When exposed to radiotherapy, pulp tissue could become less resistant, and further insults may predispose to pulp necrosis. As a result of subsequent infection, the periapical tissues could become inflammable more easily, and osteoradionecrosis could follow. Therefore, the findings in this study reinforce the need of early diagnosis of the condition of pulps within irradiated patient, and the provision of endodontic treatment when necessary, to prevent this pathway of infection with unfavourable implications for the patient.

Conclusion

Light microscopy revealed that the irradiation protocol caused significant nuclear alterations in pulp fibroblasts and odontoblasts immediately and 30 days following fractioned teletherapy; the effects were more expressive immediately after the conclusion of treatment. No such alterations were found in the control group.

The findings in TEM revealed nuclear and cytoplasmic alterations in fibroblasts of the middle third of rat incisors, only in the group of rats were killed immediately following irradiation.

Fractioned teletherapy was unable to induce inflammatory reactions, ECM hyalinization or changes in the collagen content of coronal pulp tissue in molar teeth during the study periods.

References

Anneroth G, Holm LE, Karlsson G (1985) The effect of radiation on teeth: a clinical, histologic and microradiographic study. *International Journal of Oral Surgery* **14**, 269–74.

De Moor R (2000) Influence directe et indirecte de la médication (chimiothérapie y comprise) et de l'irradiation sur la pulpe. *Revue Belge de MedecineDentaire* **55**, 321–33.

Fawzi MI, Shklar G, Krakow AA (1985) The effect of radiation on the response of dental pulp to operative and endodontic procedures: an experimental study. *Oral Surgery, Oral Medicine, and Oral Pathology* **59**, 405–13.

Hutton MF, Patterson SS, Mitchell DF, Chalian VA, Hornback NB (1974) The effect of cobalt-60 radiation on the dental pulps of monkeys. *Oral Surgery, Oral Medicine, and Oral Pathology* **38**, 279–86.

Junqueira LCU, Bignolas G, Brentani RR (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *The Histochemical Journal* **11**, 447–55.

Kalnis V (1954) The indirect effect of x-ray irradiation on the dental pulp of the dog. *Journal of Dental Research* **33**, 389–99.

Mathes SJ, Alexander J (1996) Radiation injury. *Surgical Oncology Clinics of North America* **5**, 809–24.

Matson JE, Patterson SS, Kafrawy AH, Hornback NB, Shidnia H (1978) Effect of cobalt-60 radiation on response to endodontic therapy in monkeys. *Cancer* **42**, 2581–90.

Mettler FA Jr, Upton AC (1995) Basic radiation physics, chemistry, and biology. In: *Medical Effects of Ionizing Radiation*, 2nd edn. Philadelphia, PA, USA: WB Saunders, pp. 1–29.

Meyer I, Shklar G, Turner J (1962) A comparison of the effects of 200 kV radiation and cobalt-60 radiation on the jaws and dental structure of the white rat: a preliminary report. *Oral Surgery, Oral Medicine, and Oral Pathology* **15**, 1098–108.

Nesse W, Hoekedema A, Stenega B, van der Hoeven JH, de Bont LGM, Roodenburg JLN (2006) Prevalence of obstructive sleep apnoea following head and neck cancer treatment: a cross-sectional study. *Oral Oncology* **42**, 108–14.

Nickens GE, Patterson SS, El-Kafrawi AH, Hornback NB (1977) Effect of cobalt-60 radiation on the pulp of restored teeth. *Journal of the American Dental Association* **94**, 701–4.

Sagowski C, Tesche S, Zywietz F, Wenzel S, Metternich FU (2004) The radioprotectors amifostine and sodium selenite do not modify the radiosensitivity of rat rhabdomyosarcomas. *Onkologie* **27**, 54–7.