Original Article

MICROSCOPICAL ANALYSIS OF FRACTIONATED COBALT-60 RADIOTHERAPY EFFECTS ON MANDIBLES OF RATS*

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Abstract **OBJECTIVE: The objective of the present study was to evaluate both immediate and late effects of fractionated cobalt-60 teletherapy over the mean percentage of empty osteocyte lacunae in rats mandibles. MATERIALS AND METHODS: The animals sampling (n = 45) was divided into three groups with 15 specimens each: group 1 – submitted to fractionated teletherapy (60 Gy) and sacrificed following the last irradiation dose; group 2 – the specimens were submitted to the same procedure as group 1, however were sacrificed 30 days after finishing radiotherapy; group 3 – non-irradiated specimens, control group. The radiotherapy protocol consisted of a course of 30 radiotherapy sessions, fractionated in 2 Gy/day-doses, totalling 60 Gy. After specimens perfusion with 4% paraformaldehyde, the left hemimandibles were histologically processed and 5 µm serial sections were hematoxylin-eosin stained. Duplicate calculation of the mean percentage of empty osteocyte lacunae was performed in the areas adjacent to the first and second molar roots with the Image Tool software. RESULTS: The analysis of variance supplemented with the Turkey's multiple comparison test indicated that irradiated groups 1 and 2 showed no difference, presenting higher rates of empty osteocyte lacunae (*p* = 0.005) in comparison with the control group. CONCLUSION: Fractionated cobalt-60 teletherapy causes an increase in the number of osteoplasts in bone tissues of rats' mandibles.**
Keywords: Radiotherapy; External beam radiation therapy; Late effects; Radiation damage; Osteoradionecrosis; Rats.

Resumo *Análise microscópica do efeito da radioterapia fracionada por cobalto-60 em mandíbula de rato.*
OBJETIVO: O objetivo deste estudo foi avaliar o efeito imediato e tardio da teleterapia fracionada por cobalto-60 sobre o percentual médio de osteoplastos em mandíbula de ratos. MATERIAIS E MÉTODOS: Os animais (n = 45) foram divididos em três grupos: grupo 1 (n = 15) – submetidos a teleterapia fracionada e sacrificados terminada a última dose de irradiação; grupo 2 – idêntico ao grupo 1, porém mortos 30 dias após a conclusão da teleterapia; grupo 3 (n = 15) – não-irradiado, servindo como grupo-controle. O protocolo radioterápico consistiu de 30 sessões de teleterapia, fracionadas em doses de 2 Gy/dia, totalizando 60 Gy. Após a perfusão dos animais com paraformaldeído a 4%, a hemimandíbula esquerda foi processada histologicamente. Secções seriadas (5 µm) foram coradas com hematoxilina-eosina. Selecionaram-se duas áreas próximas às raízes dos primeiros e segundos molares. O percentual médio de osteoplastos foi calculado nessa região, em duplicata, valendo-se do programa Image Tool. RESULTADOS: A análise de variância, complementada pelo teste de comparações múltiplas de Tukey, evidenciou que os grupos irradiados 1 e 2 não diferiram entre si, apresentando maiores percentuais de osteoplastos (*p* = 0,005) quando comparados com o grupo-controle. CONCLUSÃO: Concluiu-se que a teleterapia fracionada por cobalto-60, na dose estabelecida, provoca um aumento do número de osteoplastos em tecido ósseo mandibular de ratos.
Unitermos: Radioterapia; Teleterapia; Efeitos tardios; Complicações; Osteorradionecrose; Ratos.

INTRODUCTION

Radiotherapy, a modality utilized for treatment of head and neck cancer[(1)], employs ionizing radiations to control neoplastic cells, as a curative or adjuvant treatment of the malignant lesion[(2)]. The radiation dose ranges between 40 Gy and 70 Gy, and usually is fractionated in daily doses of about 2 Gy, allowing the distribution of the recommended total dose over a period of four to seven weeks[(3)].

Besides the radiation therapeutical effects, specific complications may arise following irradiation of this region, such as cavities, limitation of the mouth opening degree, decrease in the quality of the masticatory function and dysgeusia[(4,5)].

Alterations resulting from the ionizing radiation action on the bone tissue, which cause the development of osteoradionecrosis, are considered as the most severe complication from head and neck radiotherapy[(6)], and must be studied.

Marx[(7)] has defined osteoradionecrosis as a metabolic and hemostatic deficiency resulting from radiation-induced tissue injure.

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Osteoradionecrosis is characterized by the following sequence: irradiation, hypoxia, hypovascularization and hypocellularity of tissues. Irradiation affects the endothelium, and causes hyalinization and blood vessels thrombosis. The periosteum becomes fibrotic, osteocytes and osteoclasts become necrotic by fibrosis of medullary spaces[7–10]. The capacity of replacing collagen and normal bone cells in irradiated tissues is extremely affected or does not exist[7]. Because of the reduced vascularization and osteocytes destruction, osteoradionecrosis occurs in approximately 20% of patients who underwent local irradiation[3].

Baker[11] has reported the relationship between the tissues response to radiation and the capacity of repairing or not radioinduced lesions. Fast-response tissues are those which present clinical manifestation of lesions in a short time interval after irradiation like skin, mucosas, hematopoietic tissue, lymphoid tissue, and certain tumors. The slow-response tissues are those which present more delayed responses. Examples of this kind of tissue are: bone, conjunctive, muscular and nervous tissues with low proliferative activity.

Meyer et al.[12] have compared the orthovoltage (200 kV) and supervoltage (cobalt-60) effects on 70 three-month old rats, with 10 Gy, 15 Gy and 20 Gy doses. The bone tissue was studied in the interdental septum region between the first and the second lower molars. In the animals receiving orthovoltage the bone presented an irregular structure, with degenerative sites. Pyknosis was observed in osteocytes. Osteocytes presence was not evidenced on the distal septal margin, and a considerable decrease in the number of osteoblasts was observed. Medullary spaces were enlarged, showing decrease in the cellular components density. In the animals irradiated with cobalt-60, the bone was essentially normal. Occasionally, some animals showed a decrease in the number of osteoblasts and fibroblasts adjacent to the periodontal membrane.

Rohrer et al.[13] have evaluated the effects of high radiation-doses on the mandibles of eight monkeys. The animals were irradiated with a total 45 Gy dose of cobalt-60, fractionated into ten sessions over a 12-day period, equivalent to 70 Gy divided into 35 sessions over seven weeks. The animals were sacrificed within one week to six months after the treatment completion. Histological cuts were performed in the molars region (localized in the irradiation field), and in the incisives region (non-irradiated). The molars region was subdivided into cortical, Haversian and medullary bone. At each histological cut, the percentage of empty osteocytic lacunae was recorded. Osteocytes were absent in the cortical bone lacunae and in the Harvesian bone, presenting mean values of respectively 35% and 32%. Starting on the third month, the presence of empty lacunae was more remarkable. However, the values for the medullary bone were similar to those in the control group. The irradiated medullary portion of the mandibles showed a marked proliferation. The periosteum inside the irradiation field showed loss of cellularity, of vascularization, and inhibition of osteoid formation. The bone-marrow of the irradiated animals showed marked alterations, including fibrosis, proliferation of new bone tissue and endoarteritis obliterans. For the authors, the difference in percentages of empty lacunae in distinct regions of the bone tissue may be a result of the local regenerative capacity and variation of blood supply.

For Baker[11], the initial changes in the irradiated bone tissue result in a decrease in the osteocytes population. Osteoblasts tend to be more radiosensitive than osteoclasts, so after a radiotherapy course it should there be a higher disproportionality of the lytic activity. With the excessive reduction of osteocytes, bone regions become devitalized and degenerative alterations start to develop. These changes are potentialized because radiation also causes injuries to small blood vessels in the bone, as well as to the oral mucosa.

Lambert et al.[14] have reported that therapeutic radiation-doses induce death of endothelium, thrombosis and blood vessels hyalinization. The progressive vessels obliteration results in decreased microcirculation. The periosteum of an irradiated bone becomes fibrous, with destruction of osteoblasts and osteocytes, and spaces appear in the bone marrow and start being filled with fibrous tissue.

For Sykes[15], the bone cells and tissues vascularization become irreversibly damaged when irradiated, with a consequent devitalization of the bone tissue.

Based in this brief literature review, one may observe that radiation on the region of head and neck causes damages to the vascularization and affects the cellularity of the bone tissue which becomes potentially susceptible to osteoradionecrosis. Although innumerable studies in the literature have mentioned the occurrence of hypoxia, hypocellularity and hypovascularity in the irradiated bone tissue[7–10], there are few studies[13] evaluating quantitatively the alteration in the number of osteocytes in this tissue.

Therefore, the present study is aimed at evaluating the mean percentage of osteoblasts in the mandibular tissue of rats submitted to fractionated cobalt-60 radiotherapy, immediately after the completion of the radiotherapy scheme and in the subsequent 30 days, comparing the results with non-irradiated animals.

MATERIALS AND METHODS

The present study had its protocol approved by the Scientific Committer for Ethics of Faculdade de Odontologia da Universidade Luterana do Brasil, Canoas, RS, Brazil.

The study sample included 45 male, 80-day old albine rats (*Rattus norvegicus*), of the Wistar strain, originating from the biotery of Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, and weighting 220–290 g at the beginning of the experiment. During the experimental period, the animals received Labina-type solid diet and water *ad libitum* and were randomly distributed into three groups: group 1 (experimental group 1) – 15 animals submitted to 60 Gy radiation-dose, and sacrificed at the end of the radiotherapy; group 2 (experimental group 2) – 15 animals submitted to 60 Gy radiation-dose, and sacrificed 30 days after the radiotherapy completion; group 3 (control group) – 15 non-irradiated animals, subdivided into two groups with, respectively, seven rats for the experimental group 1 (group 3a), and eight for the experimental group 2 (group 3b).

The animals in groups 1 and 2 were irradiated in the Service of Radiotherapy at Hospital São Lucas – Pontifícia Universidade Católica do Rio Grande do Sul, RS, Brazil, employing a cobalt-60 teletherapy unit with rotational gantry (Philips, XK 5101 model), with 1.25 MeV energy. The total dose employed was 60 Gy, divided into 30 2Gy-fractions, delivered during 15-minute daily sessions, from Mondays to Fridays, over a 6-week period[16,17]. The distance between the radiation beam emitter and the animals skin surface was 60 cm (Figure 1A). The head and neck region was placed in the irradiation field measuring 20 cm × 20 cm, with the animals immobilized during the session by means of plastic devices (Figure 1B).

Animals of groups 1 and 3a were sacrificed 60 hours after the teletherapy completion. Animals of groups 2 and 3b were equally sacrificed 30 days after. After intraperitoneal anesthesia with sodic tiopental and S+ ketamine hydrochloride at 22 mg/kg, the animals were perfused with 4% paraformaldehyde. Previously to the employment of the fixative solution, the animals head circulatory system was washed with 50 ml 0.1 M, 7.3 pH phosphate buffered saline solution

The left hemimandible of the animal was dissected, and a fragment containing the three molar teeth and the middle third of the incisive was submitted to evaluation by light microscopy. The samples were decalcified in ethylenediamine acetic acid at 17% (pH 7.0). The specimens were processed by the routine technique, taking care to keep the plane formed by long axis of the three molar teeth parallel to the plane of one of the block surfaces. The histological sheets were hematoxylin-eosin stained and three serial 5 µm thick cuts were obtained.

The site of evaluation in the histopathological cuts was the medullary bone, near the roots of the first and second lower left molars.

Two areas of each histological sheet were selected, corresponding to the regions of first and second molars. Images capture was performed with an Olympus AX-70 optic microscope coupled with a system of digital images capture, with 200x magnification. The software Image-Pro Plus version 4.0 was utilized for the images storage.

Images were entered into the ImageTool software, for osteocytes and osteoplasts counting (Figure 2) by means of the tag and count resource. The image was divided into quadrants to be magnified in order to facilitate the counting. This count was repeated twice for each image. Numeric values were recorded in tables. Afterwards, an arithmetic mean of the two counts was calculated.

Aiming at evaluating the interexaminer concordance between the first and the second counts, the intraclass correlation coefficient was utilized. The variance analysis, supplemented by the Tukey's multiple comparison test at 5% significance level, was employed to evaluate the difference between the groups concerning the percentage of osteoplasts in tissues, with the SPSS software version 8.0.

RESULTS

There was no animal death during the radiotherapy scheme. One animal in group 2 died during the 30-day interval, the waiting-time between the radiotherapy completion and the euthanasia of the animals in this group.

During the treatment, the animals presented with a debilitated appearance. Some of them demonstrated a visible decrease in the quantity of hair on the head, i.e., the radiation portal area, a fact more clearly observed in the animals of group 2 which were kept alive up to 30 days after the radiotherapy completion.

Figure 1. **A:** Radiotherapy Philips XK 5101 model equipment. Animals placed on the equipment with the head at a 60 cm distance from the radioactive source. **B:** Animals positioned on a wooden device with slots, inside the plastic restrainer.

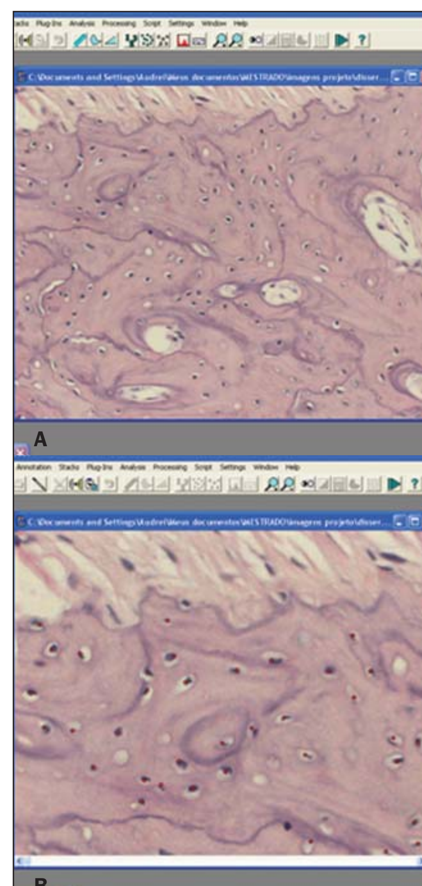

Figure 2. **A:** Selected image. **B:** Osteocytes and empty osteocytic lacunae being marked and counted.

The intraclass correlation coefficient demonstrated a good reproducibility between the first and the second measurement (r_i = 0.956; $p < 0.001$).

The animals in groups 1 and 2 (irradiated) did not differ from each other, presenting higher rates of osteoplasts (Figures 3 and 4) when compared with group 3 (control) (Figure 5) (Tables 1 and 2), according to the variance analysis supplemented by the Tukey's multiple comparison test at a 5% significance level.

The nuclei of some endothelium and osteocytes cells of the irradiated animals presented alterations similar to vacuoles or bubbles, or a spherical, slightly stained region (Figure 3).

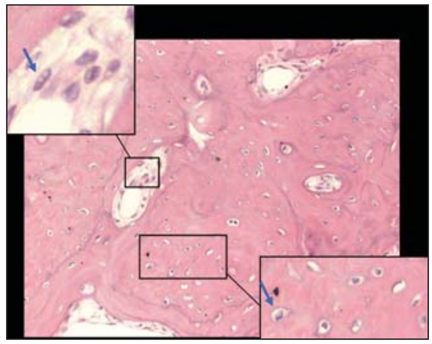

Figure 3. Photomicrography of mandibular bone tissue of an animal in group 1. Highlighted regions on upper left and lower right corners show respectively presence of nuclear alteration in endothelium and osteocytes. (Hematoxylin-eosin, 200× magnification).

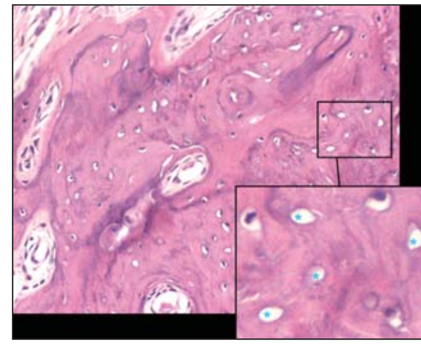

Figure 4. Photomicrography of mandibular bone tissue of an animal in group 2. The highlighted region shows the presence of osteoplasts (asterisks). (Hematoxylin-eosin, 200× magnification).

DISCUSSION

A concern that was present during the pilot project of this experiment was the necessity, or not, of anesthesia of the animals during the radiotherapy sessions for them to keep their heads towards the radiation area. However, while the anesthetic procedure aided in the restrain or displacement of the rats, a high number of applications could induce their death[13,18]. Consequently there would be a decrease in the size of the sample, considering that the protocol defined 30 radiotherapy sessions. Fractionated radiotherapy has been utilized in many studies with animals without anesthesia during treatment sessions[19–22]. On the other hand, studies performed with monkeys by Hutton et al.[23], Nickens et al.[24] and Rohrer et al.[13], followed a protocol establishing that the anesthetic procedure was performed at each radiation dose. Even the studies of Sweeney et al.[25], Zywietz et al.[26] and Sagowski et al.[16,17] utilizing rats as animal models share this methodology.

Although employing a single 15 Gy x-radiation dose in rats, English et al.[27] were the first authors to describe the form of animals immobilization during the radiotherapy, without using anesthesia. The animals were placed inside a cylindrical tube whose anterior extremity had a hole with 1.0 cm–1.5 cm to allow the insertion of the animal nose. After that, a rubber lid with a tail slot was placed on the posterior extremity of the tube. The methodology employed by these authors and by English[28] was a model utilized for idealization of PET mineral water bottles used as restrainers for the animals in the present study, eliminating the necessity of anesthesia in the radiotherapy sessions. However, differently from the above mentioned authors, the apparatus developed in this experiment allowed the exposure of the rat head through the anterior orifice of the bottle, facilitating the placement of this region inside the irradiation field.

A constant concern along the development of the present study was the conformity of the experimental design with the clinical reality. So, the radiotherapy dose employed in the animal model was the same employed in the protocol for treatment of oral neoplasms in humans[3]. However, there was a doubt if the rat, as a function of its lower body volume compared to the oncological patient, could support the usual radiotherapy dose. But, analyzing the current literature, one may observe that doses of at least 60 Gy have been fractionatedly utilized in rats, employing different therapy modalities such as x-radiation[19,21,22], cobalt-60[16,17,26] and the linear accelerator[20]. In the present experiment, the same protocol described by Sagowski et al.[16,17] was employed, i.e., 30 sessions of cobalt-60 in 2 Gy-doses, totalling 60 Gy fractionated from Mondays to Fridays over a six-week period, which, in the final analysis, is the radiotherapy scheme utilized for treatment of oral cancer in humans.

Radiotherapy, notwithstanding its beneficial effects on the tumor tissue localized

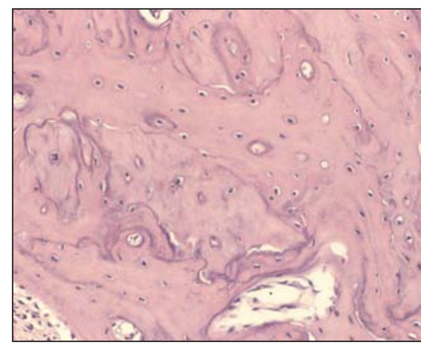

Figure 5. Photomicrography of mandibular bone tissue of an animal in group 3 (non-irradiated). (Hematoxylin-eosin, 200× magnification).

in the head and neck region, causes injuries to normal tissues localized in the radiation portal. So, salivary glands, oral mucosa, bone, teeth, masticatory muscles and temporomandibular joints are affected by ionizing radiation[5]. The teletherapy effects on the dental pulp and submandibular gland could be observed in the studies of Vier-Pelisser et al.[29] and Vier-Pelisser[30].

However, amongst radiotherapy late complications, osteoradionecrosis remains as a significant and serious clinical problem[31,32], because the irradiated bone presents hypoxia, hypovascularization and hypocellularity[7–10]. Despite the comments of several authors on the destruction of osteocytes in irradiated bone tissues[3,11,33], few studies[13] have performed a quantitative analysis of the reduction of these cells in the bone.

The osteocyte occupies in the bone matrix a compartment denominated osteocytic lacuna or osteoplast[34]. When this cell

Table 1 Mean percentage of osteoplasts in hematoxylin-eosin stained mandibular bone tissue of rats in experimental control groups.

Group	N	Percentage of osteoplasts			
		Mean	Standard deviation	Minimum	Maximum
G1	14*	12.69^A	3.95	5.92	18.83
G2	12*	12.93^A	4.71	3.53	20.67
G3	12*	8.03^B	2.85	3.08	13.95

* One, three and three specimens, respectively in groups 1, 2 and 3, were lost during the histological processing. *Note 1:* Means followed by distinct letters differ significantly in the variance analysis supplemented by the Tukey's multiple comparison test, at a significance level of 5%. *Note 2:* Groups 3a and 3b were jointly statistically analyzed.

Table 2 Variance analysis of the mean percentage of osteoplasts in hematoxylin-eosin stained mandibular bone tissue of rats in experimental control groups.

Variance cause	Freedom degree	Square sum	F	P
Group	2	186.51	6.08	0.005
Experimental error	35	536.67		
Corrected total	37	723.18		

necrotizes, the osteocytic lacuna becomes empty. So, the objective of the present study was to microscopically evaluate the mean percentage of osteoplasts in mandibular bone tissue of rats submitted to fractionated cobalt-60 radiotherapy.

Stone et al.[35] reported that the chronic radiation effects can be observed over months to years after the therapeutic scheme completion. Taking this fact into consideration, the present study sought to evaluate the effects of fractionated radiotherapy on mandibles of rats over two experimental periods: the first, immediately after the radiotherapy scheme completion, and the second, after the 30 subsequent days, since the tissue metabolism of rats is faster than the humans'.

The results of this investigation demonstrated that the percentage of osteoplasts in booth experimental groups, independently from the observation period, was statistically higher than the percentages in the control groups. This confirms innumerable authors assertions[3,7,9–13,22,33] reporting a hypocellularity condition in the tissue exposed to radiation.

In this experiment, the decrease in the number of cells in the irradiated mandibular tissue was observed both immediately after the teletherapy completion and at the 30 subsequent days. It should be expected, however, that the percentage of osteoplasts would be higher in the experimental group 2, compared with group 1, since osteoradionecrosis is a late effect of irradiation. Other studies employing observations over longer periods are necessary to evaluate a possible worsening of the hypocellularity in this tissue.

Considering the results of this research, one may ask the reason for the increase in the presence of osteoplasts in the irradiated tissue. Maybe, the damage observed on the terminal vascular bed, causing injury to the local microcirculation and to the support stroma characterized by telangiectasia and occlusion of blood capillary vessels[11,14,33], causes a decrease in the vascularization[36], i.e, in the blood supply to osteocytes which end up necrotizing. The injury in the local microcirculation also could be responsible for a decrease in the quantity of oxygen in the bone tissue. Additionally, the microcirculation seems to be more problematic in the mandible than in the maxilla. The latest typically presents more vascularized than the mandible, so the osteoradionecrosis incidence is higher in the mandibular than in the maxillary tissue[31,37].

In spite of not being the objective of the present study, one could observe both in osteocytes and endotelial cells present in the field of study, nuclear alterations characterized by the appearance of slightly stained regions similar to bubbles or vacuoles. These results corroborate the presence of damage from the irradiation to osteocytes and vascular endothelium. These same nuclear alterations were observed in pulpal fibroblasts of molars in cobalt-60-irradiated rats[30].

The decrease in the number of osteocytes in the irradiated mandibular tissue of rats characterized by the increase in the presence of osteoplasts would help to explain the occurrence of osteoradionecrosis. Shafer et al.[33] reported that, in general, radiation, trauma and infection are factors involved in the pathogenesis of this significant complication from radiotherapy in the head & neck region. The recent literature indicates a mechanism of fibroatrophy for development of osteoradionecrosis to the detriment of the traditional mechanism of vascular insufficiency[32,38]. Another hypothesis is that irradiation would lead to osteoradionecrosis by apoptosis induction in bone cells[39]. It is thought that the decrease in the number of osteocytes in the irradiated bone tissue is a significant component in the etiology of osteoradionecrosis.

CONCLUSION

Based on the methodology employed in the present study and respective results, one may conclude that cobalt-60 teletherapy is capable of promoting a statistically significant increase ($p = 0.005$) in the mean percentage of osteoplasts in mandibular bone tissue of rats. This increase is observed both immediately after the completion of the radiotherapy scheme and in the subsequent 30 days.

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