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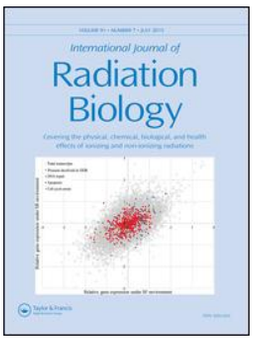

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RESEARCH ARTICLE

Ameliorative effect of black grape juice on systemic alterations and mandibular osteoradionecrosis induced by whole brain irradiation in rats

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ABSTRACT

Purpose: Whole brain irradiation (WBI) causes a variety of secondary side-effects including anorexia and bone necrosis. We evaluated the radiomodifying effect of black grape juice (BGJ) on WBI alterations in rats measuring food and water intake, body weight, hemogram, and morphological and histological mandibular parameters.

Materials and methods: Forty male rats (200–250 g) were exposed to eight sessions of cranial X-ray irradiation. The total dose absorbed was 32 Gy delivered over 2 weeks. Four groups were defined: (i) NG: non-irradiated, glucose and fructose solution-supplemented (GFS); (ii) NJ: non-irradiated, BGJ-supplemented; (iii) RG: irradiated, GFS-supplemented; and (iv) RJ: irradiated, BGJ-supplemented. Rats received daily BGJ or GFS dosing by gavage starting 4 days before, continuing during, and ending 4 days after WBI.

Results: RJ rats ingested more food and water and showed less body weight loss than RG rats during the irradiation period. Forty days after WBI, irradiated animals started losing weight again compared with controls as a consequence of masticatory hypofunction by mandibular osteoradionecrosis (ORN). Osteoclastic activity and inflammation were apparent in RG rat mandibles. BGJ was able to attenuate the severity of ORN as well as to improve white and red blood cell counts.

Conclusions: Fractionated whole brain irradiation induces mandibular changes that interfere with normal feeding. BGJ can be used to mitigate systemic side-effects of brain irradiation and ORN.

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Black grape juice; cranial irradiation; mandible; osteoradionecrosis; rat

Introduction

Radiotherapy (RT) is a strategy for brain tumor management, including gliomas (Sarmiento et al. 2015), meningiomas (Mehdorn 2016), and brain metastases (Scoccianti and Ricardi 2012). RT is often effective at producing durable tumor responses, but inadvertently produces several debilitating late side-effects, including neurological impairments, anorexia, hematological disturbances, and osteoradionecrosis (ORN) (Lee et al. 2012). Anorexia can be mistakenly diagnosed in brain tumors (Madhusoodanan et al. 2010) within the context of appetite loss and malnourishment, mostly related to oral cavity disorders (Robinson et al. 2001; Jham and da Silva Freire 2006).

Facial ORN appears in patients who were treated with RT for head or neck cancer. The mechanism of this pathology is not clear and can be manifested years after RT. Infections, trauma and dental procedures can initiate necrosis on face bones with the mandible being frequently affected (Thorn et al. 2000). Tamplen et al. (2011) developed an experimental rat model of mandibular ORN. They induced ORN by mandibular removal of teeth 7 days after a 20 Gy high dose rate of brachytherapy. Histopathological studies revealed the presence of fibrosis and inflammation along with a reduction of bone formation in comparison to control rats (Tamplen et al. 2011).

All disorders relative to ionizing radiation are ultimately related to oxidative processes. Antioxidants are often explored to mitigate tissue damage induced by X-rays and gamma rays. Isolated antioxidant compounds, functional foods, or medicinal plants are administered to the animals in experimental models of radiation sickness (Saada et al. 2009; Mansour and Tawfik 2012; de Freitas et al. 2014). Regarding mandibular ORN treatments, antibiotic administration, hyperbaric oxygen therapy, and procedures such as surgical debridement are often employed to reduce disease morbidity (Thorn et al. 2000). A promising strategy to prevent radiation injury non-invasively uses antioxidant supplementation for the management of secondary effects of fractionated whole brain irradiation. Grape-derived products such as seeds extract and juice have positive radiomodifying effects on

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injuries induced by irradiation (Ramos de Andrade et al. 2009; Saada et al. 2009; de Freitas et al. 2014). Previous studies have shown that black grape juice (BGJ) is rich in resveratrol and quercetin (de Freitas et al. 2013). Our team has explored the effects of BGJ on hematological parameters, spleen and cardiac damage on experimental models of radiation sickness (Ramos de Andrade et al. 2009; de Freitas et al. 2013; 2014).

The present study evaluates the radiomodifying effect of BGJ on side-effects induced by whole brain irradiation on rats through the measurement of food and water intake, body weight, hemogram, and morphological and histological mandibular parameters.

Materials and methods

Black grape juice (BGJ)

BGJ was produced from organically grown grapes of *Vitis labrusca* species. Our team previously quantified selected polyphenols (8.4 mg/l gallic acid, 5.2 mg/l catechin, 31.3 mg/l resveratrol, 18.2 mg/l caffeic acid, 8.0 mg/l ellagic acid, 18,7 mg/l quercetin, and 8.7 mg/l kaempferol) in BGJ by high-performance liquid chromatography (HPLC) (de Freitas et al. 2013). An isocaloric solution (GFS) was prepared using an equimolar mixture of glucose and fructose to have the same sugar composition present in the BGJ (180 g/l).

Animals

Forty male Wistar rats (Harlan, Barcelona, Spain), weighing 200–250 g at the beginning of the study, were kept in standard wire-topped makrolon® polycarbonate type III cages containing two animals each, with sawdust bedding, on a 12-h light/dark cycle (lights on at 08:00 h) at room temperature. The animals were fed *ad libitum* with standard rodent chow (Panlab, Barcelona, Spain) and were given unrestricted access to water. All animal procedures followed protocols approved by the Committee on Care and Use of Experimental Animal Resources from University of León, León, Spain, and were in accordance with the indications of the current Spanish and European laws (RD 53/2013 and EU Directive 2010/63/EU).

Whole brain irradiation and experimental design

Before whole brain irradiation (WBI), all animals were anesthetized with a mixture of xylazine and ketamine (7.5/60 mg per kg, i.p.) for immobilization purposes. The animals were placed in *decubitus pronus* on a Plexiglas board, and four animals were irradiated at the same time. The animals were placed with their bodies underneath cerrobend shielding plates (1.5 cm thick, blocking 99.87% of radiation), so that only the heads were exposed to X-rays (with eyes and nose also protected). Eight fractions of 4 Gy with a source-skin distance of 36 cm (0.53 Gy/min) were delivered at noon over 2 weeks (4 daily fractions per week), as previously described (Madsen et al. 2003). The rats were divided randomly into four groups: NG: non-irradiated, glucose and fructose solution-supplemented (GFS); NJ: non-irradiated, BGJ-supplemented; RG: irradiated, GFS-supplemented; and RJ: irradiated, BGJ-supplemented. The BGJ or GFS was administered by gavage (0.5 ml per 100 g body weight) daily starting 4 days before X-rays exposure, continuing during WBI, and ending 4 days after WBI procedure (Figure 1). The X-ray apparatus was a Maxishot 200 (200 kV, 4.5 mA, ceramic anode, YXLON International GmbH, Hamburg, Germany) operated by qualified staff (Instrumental Techniques Laboratory, University of León) in accordance with Spanish legislation on radiation equipment. X-ray filtration was accomplished in the Maxishot 200 machine following manufacturer's instructions using 4-mm thick beryllium and 3-mm thick aluminum filters in the X-ray tube. Uniform irradiation of the brain was assessed via depth-dose deposition curves (PDD) and analysis of the radiation beam homogeneity. The X-ray beam half value layer was 0.8 mm Cu, assuring 5% uniformity in dose deposition

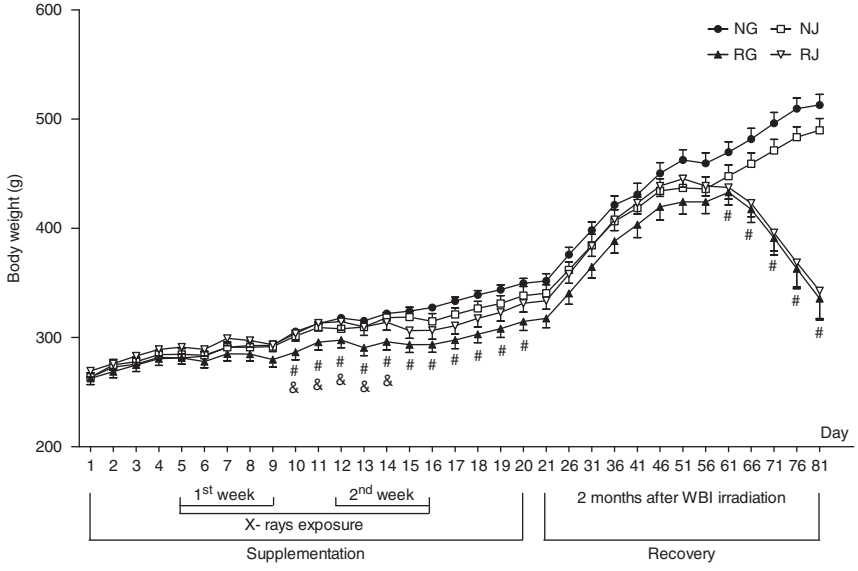

Figure 1. Growth curves for groups NG (non-irradiated; supplemented with glucose and fructose), NJ (non-irradiated; supplemented with black grape juice), RG (irradiated; supplemented with glucose and fructose), and RJ (irradiated; supplemented with black grape juice). Data are means ± SEM from 10 animals per group. #$p < .05$ from NG and $p < .05$ from RG.

until 1.5 cm depth. Using radiochromic film, radiation beam homogeneity was studied in the coronal plane of the head, with 5% homogeneity in both rostral-caudal and left-right axes. Dose uncertainty with coverage factor k = 2 was 10%. Maximum dose achieved at the lower mandible was estimated to be 4 Gy.

Body weight, food and water intake

Body weight, drinking and feeding data were obtained at the beginning of BGJ or GFS supplementation until 2 months after the last brain irradiation session. Food and water intake per cage were estimated by the difference of the weight of the cage lid containing food pellets and the water bottle weight from one day to the next. The quantity of food and water were normalized by rat body weight according to the following formula: (weight of rat 1/mean of rat 1 and rat 2 weights) × (total food (g) or water intake (ml) consumed/2), where rat 1 and rat 2 are the two animals sharing a cage. Graphs of body weight, food and water intake show the data collected between day 1 and day 20. After day 21, Figures 1 and 2 show the data every 5 days to the end of the study, 2 months after the last brain irradiation session.

Euthanasia and samples collection

Two months after the last dose of WBI, the rats were killed by exsanguination under deep anesthesia induced by i.p. injecting 200 mg/kg body weight pentobarbital. The carotid was cannulated and blood samples were collected in tubes containing EDTA. The lower mandible was dissected and preserved in 10% buffered-formalin. One hemi-mandible was used for morphological measurements and the other was reserved for histopathological studies.

Mandibular macroscopic measurements

The mandibles were boiled for 2 min in water for the soft tissue to be separated. The remaining tissue present on mandibles was removed by scraping with a brush. After this procedure, the hemi-mandibles were photographed by a digital camera (Lumix DMC-TZ30,

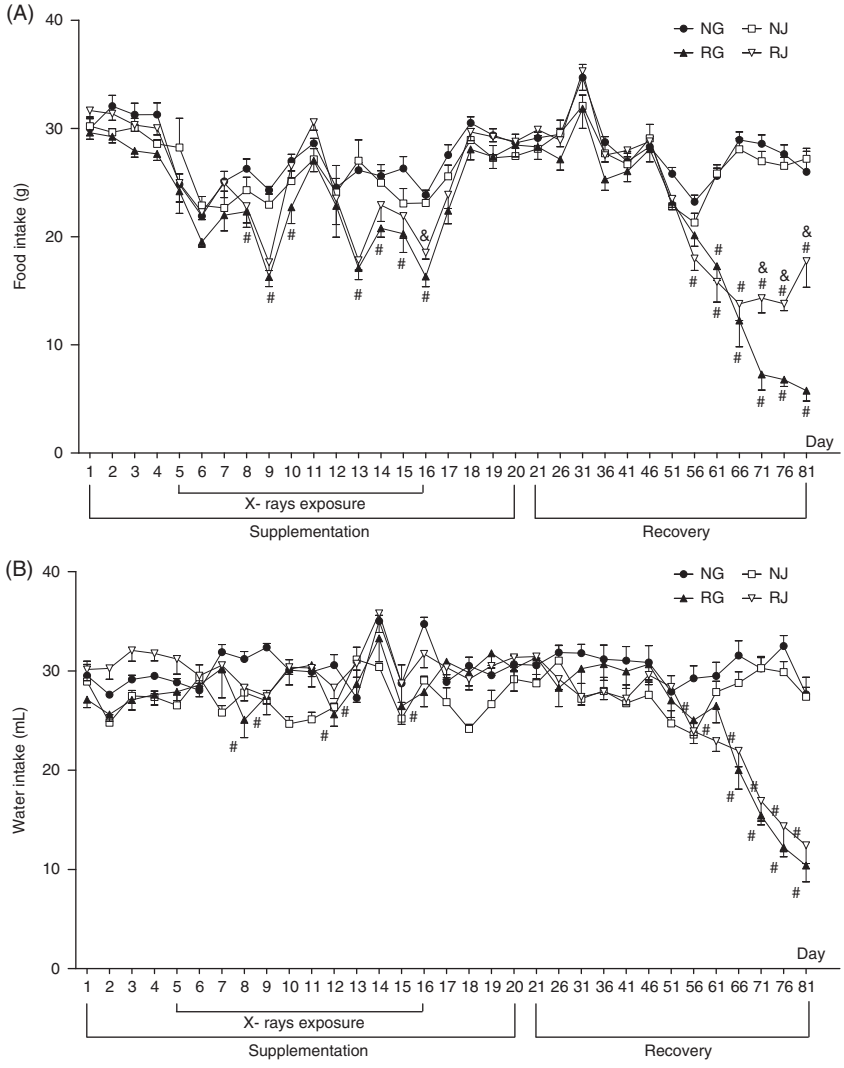

Figure 2. (A) Food intake (g) and (B) water intake (ml) from groups NG (non-irradiated; supplemented with glucose and fructose), NJ (non-irradiated; supplemented with black grape juice), RG (irradiated; supplemented with glucose and fructose) and RJ (irradiated; supplemented with black grape juice). Data are means ± SEM from 10 animals per group. #$p < .05$ from NG and $p < .05$ from RG.

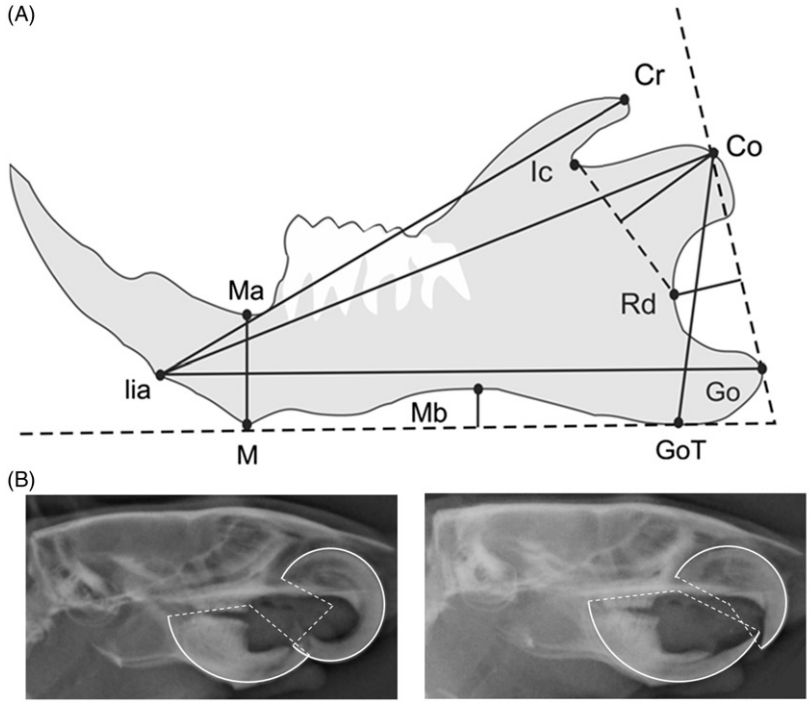

Figure 3 Mandibular points and measurements: Mandibular length I (Co-Ia); Mandibular body length (Go-Ia); Mandibular length II (Cr-Ia); The depth of mandibular curvature (Rd at right angle to Co-Go); Mandibular head (Co at right angle to Rd-Ic); Mandibular base depth (Mb at right angle to GoT-M); Ramus height (Co-GoT); Mandibular body height (M-Ma); Gonial angle (intersection between Co-Go and GoT-M). Adapted from Guerreiro et al. (2013). (B) Representative head radiographs of a non-irradiated rat (NG group, right) and an irradiated rat (RG group, left) showing the circle sector approach used to estimate incisor angulations.

Panasonic Corp, Osaka, Japan). The relative length of the upper and lower incisors was estimated as angulation measurements on whole-head radiographs, assuming that the shape of each teeth's anterior edge was a circle sector and measuring the angle between the center of the corresponding circle, the eruption point and the incisor tip (Figure 3(B)). Macroscopic measurements were carried out on all pictures using the image analysis package Digimizer® 4.1.1.0 (MedCalc Software bvba, Ostend, Belgium).

The biometric points used in this study are based on the work by Guerreiro et al. (2013) and are described as follows (Figure 3):

1. Menton (M) is the lowermost point situated on the mental symphysis border.
2. Alveolar point of the mandibular incisor (Ia) is the lowest point situated on the buccal alveolar bone border of the mandibular incisor.
3. Mandibular alveolar point (Ma) is the deepest point of the upper part of the alveolar crest between the first molar and mandibular incisors.
4. Condylion (Co) is the most superior and posterior point of mandibular head.
5. Gonion (Go) is the most posterior point of the mandibular angle contour.
6. Gonial tangent (GoT) is the lowest point of the mandibular angle contour.
7. Coronoid (Cr) is the most posterior and superior point of coronoid process.
8. Ramus depth (Rd) is the deepest point in the concavity of mandibular ramus.
9. The point located in the notch between the mandibular head and coronoid process (Ic).
10. Mandibular base (Mb) is the deepest point of the mandibular base concavity.

For sagittal measurements, the mandibular length I (ML I) was obtained by the distance between Co and Ia points (Co-Ia). The mandibular body length (MBL) was the distance between points Go and Ia (Go-Ia). The mandibular length II (ML II) was the distance between points Cr and Ia (Cr-Ia). The depth of mandibular curvature (MRCD) was the distance from the Rd point at right angle to the Co-Go line (Rd at right angle to Co-Go). Mandibular head (MH) was the distance from point Co at right angle to the Rd-Ic line.

In the vertical direction, the mandibular base depth (MBD) was obtained by the distance from point Mb at right angle to the GoT-M line (Mb at right angle to GoT-M). The ramus height (RH) was calculated by measuring the distance between Co and GoT (Co-GoT). Finally, the mandibular body height (MBH) was obtained by the distance between M and Ma points. The measurement of the gonial angle (GA) was obtained by the intersection of lines passing through points Co-Go and GoT-M.

Morphometric analysis

The whole rat mandibles were scanned by high-resolution micro-computed tomography (SkyScan 1174, SkyScan, Kontich, Belgium). The small sample-holder device for μCT was used to fit the specimen with the long axis

perpendicular to the floor of the specimen holder and the X-ray source. Images were obtained by 50 kV X-ray tube voltage and 800 μA. All specimens were scanned using 1 mm aluminum filter and at 16.7 × 16.7 μm pixel size resolution. For each specimen, a series of 620 projection images were obtained with a rotation step of 0.3° and frame averaging 2 for a total 180° rotation. The scanning time for each sample was approximately 4 h using an exposure time of 11000 ms. Flat field correction was performed at the beginning of each scan.

The images obtained during scanning were reconstructed using the software NRecon (SkyScan). The correction values for attenuation coefficient, beam hardening, smoothing and ring-artifact reduction were the same in all samples.

For morphometric analysis in 3D, the software provided by the manufacturer (CTAn, Bruker microCT, Belgium) was used. The region of interest was manually delimited in each of the samples, from the cortex at the base of the horizontal branch of the mandible to the root of the lower incisor. Global grayscale threshold levels for this area were between 64 and 250. Morphometric analysis was based on the 2-D and 3-D internal CTAn plug-ins. This analysis was performed in the coronal plane. The morphometric parameters examined were bone volume/tissue volume ratio (bone volume fraction, BV/TV), cortical thickness (Ct.Th), cortical bone porosity (Ct.B.Po) and cortical pore diameter (Ct.Po.Dm).

Hematological parameters

The hematological parameters analyzed were: white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), plateletcrit (PCT), mean platelet volume (MPV), and platelet distribution width (PDW). All analyses were performed using an automatic counter (Diatron Abacus Junior Vet, Diatron Lab, Austria).

Histologic analysis

The hemi-mandibles were decalcified in 10% EDTA (pH 7.4) for 7 days, fixed in formalin and embedded in paraffin. Sagittal sections of 4 μm were obtained with a standard microtome and were stained with hematoxylin and eosin (H&E). All samples were analyzed by a pathologist who did not know about the experimental protocol. The fields under the microscope were captured by WinTV® software.

Statistical analysis

Data were analyzed using two-way analysis of variance (ANOVA; 2 whole brain irradiation [WBI] levels ×2 black grape juice [BGJ] supplementation levels) by Statistica 10.0 software (Statsoft Inc, Tulsa, OK). The Bonferroni *post-hoc* test was used when appropriate. Data from incisor angulation estimations were analyzed using the Wilcoxon matched pairs signed ranks test. Values were expressed as means ± SEM. A value of $p < .05$ was considered statistically significant. Graphs were created using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

This work was prepared in accordance with the ARRIVE guidelines for animal research (Kilkenny et al. 2010).

Results

Body weight, food and water intake

Rats gained weight before irradiation as expected (days 1–4) (Figure 1). By day 10 (one day after the 4th session of cranial irradiation) irradiated rats had lost 6.2% body weight in comparison to non-irradiated (NG) rats. Significant body weight change was noticed from day 10 to day 20 in the group of rats irradiated (RG group) (Figure 1). In contrast, rats supplemented with BGJ (RJ) showed a body weight decrease of 5.6% on day 10 (Figure 1). In addition, BGJ supplementation protected the irradiated animals from radiation-induced anorexia from day 10 to day 14 (Figure 1). However, on days 15 and 16, RG and RJ lost weight in the same proportion in relation with NG. The whole brain irradiation (WBI) procedure finished on day 16 (8th session of WBI). The supplementation with juice or glucose and fructose solution was maintained for 4 more days (days 17–20) after the last brain irradiation. Irradiated rats lost weight until day 21 when compared to the non-irradiated group (NG). The growth curve shows that irradiated rats gained weight similar to the others approximately until day 61. In any case, the growth of rats in the RG group was apparently less than NG, NJ and RJ groups, but it was not significant until day 61 (Figure 1). The weight loss range for the RG group in comparison to the non-irradiated group (NG) for days 61, 66, 71, 76 and 81 was 7.9%, 13%, 22%, 29 and 35%, respectively. The body weight for control groups (NG and NJ) increased during the course of the study. Irradiated rats supplemented with BGJ (RJ) lost less body weight than the irradiated control group (RG), albeit no significant differences were found.

Regarding food intake, all animals had similar feeding behavior until day 8 (the 3rd WBI session) (Figure 2(A)). During the WBI irradiation procedure, there was statistical difference between NG and RG groups on day 8, day 9 (4th WBI session), day 10, day 13 (6th WBI session), day 14, day 15 (7th WBI session), and day 16 (the 6th session of WBI). From day 26, RG animals showed a diminished food intake until euthanasia when compared with non-irradiated animals (NG) (Figure 2(A)). A significant, progressive decrease of food intake was observed in RG animals from day 56 until euthanasia (day 81) in comparison to NG rats. The feeding behavior for RG and RJ animals was not statistically different during most of the days, but we observed a tendency for RJ animals to eat more than RG rats. There was statistical significance in food intake comparing RG and RJ groups on days 66, 71, 76 and 81. There were no statistical differences between RJ and NG animals for food intake during the experiment, except on days 56, 61, 66, 71, 76 and 81. Before the first whole brain irradiation (WBI) session, drinking activity was very similar for all groups. Until day 7, we did not observe differences in water intake for irradiated rats. On day 8, acute effects of

radiation began to appear. Statistical differences were detected between the non-irradiated control group (NG rats) and the irradiated control group (RG) on days 8, 9, 12, and 16 in the WBI period (Figure 2(B)). The water intake for RG rats was lower than NG rats on these days. The irradiated rats (RG) continued drinking less again starting on day 56 until the end of the study. Irradiated and juice-supplemented (RJ) rats drank less water than rats in the NG group on days 61, 66, 71, 76 and 81. There was a tendency for irradiated and juice-supplemented (RJ) rats to drink more water than rats in the RG group (Figure 2(B)).

Mandibular measurements

With regard to mandibular macroscopic measurements (Table 1), a significant decrease between groups was found in the three sagittal measurements, mandibular length I (ML I), mandibular body length (MBL), and mandibular length II (ML II) for the irradiated control group (RG) as follows: ML I decreased by 9.07% for RG in comparison to the control group (NG). Black grape juice (BGJ) was able to increase by 5.06% the ML I parameter relative to that in the RG group. The total whole brain irradiation (WBI) reduced the MBL value by 12.38% in comparison to that in the NG group. In contrast, BGJ increased MBL by 4.92% relative to that in the RG group. ML II was affected by irradiation, suffering a reduction of 9.59% in respect to controls. In this case, BGJ was not able to increase the parameter value. Neither the remaining sagittal parameters nor gonial angle (GA) were affected by radiation. The only vertical parameter which was modified by irradiation was the ramus height (RH). There was a 5.55% decrement in this dimension in the RG group in comparison to that in the NG group. BGJ increased by 6.31% the RH value in relation to RG group. There was no difference in RH between NG and RJ groups, which indicates that BJG completely restored this parameter.

Incisor angulation was markedly different in irradiated animals when compared to sham-irradiated controls. Based on radiographs taken at the time of euthanasia, the upper incisor angulation (UIA, Table 1) was significantly higher in RG rats than in NG animals, and BGJ supplementation induced a significant decrease in UIA, yet still higher than the upper incisor development of control rats. The lower incisor angulation was non-significantly lower in both irradiated groups in respect to controls. Two RG rats and one RJ suffered lower incisor fracture and were excluded from the analyses.

Morphometric analysis

A significant reduction in cortical bone thickness was apparent in the irradiated control group (RG) compared to that in the non-irradiated control group (NG). However, no clear effects of irradiation were apparent in the remaining morphometric parameters, other than a tendency to increase cortical pore diameter and cortical bone porosity, BV/TV being non-significantly lower in RG than in NG animals (Figure 4). As a result of BGJ supplementation, a trend can be seen for cortical thickness and bone porosity to increase.

Histopathology analysis

Normal osteoclastic and osteoblastic activity was observed in the mandibles of control rats (NG and NJ) (Figure 4(A), (B)). There was an increase in osteoclastic activity in mandibles of irradiated control rats (RG) (Figure 4(E)). Cranial irradiation increased the number of osteoclasts in the mandibles of irradiated control rats (RG) (20.75 ± 0.24) in comparison to non-irradiated, glucose/fructose-supplemented rats (NG) (10.75 ± 0.96). The number of osteoclasts present in mandibles from irradiated and BGJ-supplemented rats (RJ) was lower than the count found in RG group (7.50 ± 0.48) (Figure 4(E)). There were no differences in histopathology parameters between the non-irradiated control (NG) group and the non-irradiated, BGJ-supplemented (NJ) group. The NJ group yielded the highest number of osteoblasts in the mandible in comparison to the other groups (Figure 4(F)); the values found for NG, NJ, RG, and RJ groups were 218.25 ± 0.59, 330.25 ± 0.94, 230.00 ± 0.41 and 193.25 ± 0.55, respectively. In the NG and NJ groups, necrotic zones were absent in the bone (Figure 4(G)). Irradiated rats (RG) showed $3.43 \pm 0.09\ mm^2$ of necrotic area, while irradiated and supplemented rats showed significantly less necrosis

Table 1. Biometric mandibular measurements from the experimental groups NG (non-irradiated; supplemented with glucose and fructose), NJ (non-irradiated; supplemented with black grape juice), RG (irradiated; supplemented with glucose and fructose) and RJ (irradiated; supplemented with black grape juice).

Measurements	NG	NJ	RG	RJ
ML I (mm)	27.79 ± 0.44	26.94 ± 0.24	$25.27 \pm 0.25^*$	$26.55 \pm 0.40\dagger$
MBL (mm)	$28.51 \pm 0,49$	27.11 ± 0.68	$24.98 \pm 0.21^*$	$26.21 \pm 0.48\dagger$
ML II (mm)	24.82 ± 0.59	$24.30 \pm 0,71$	$22.44 \pm 0.27^*$	23.47 ± 0.40
MRCD (mm)	4.08 ± 0.18	3.64 ± 0.40	3.58 ± 0.33	3.48 ± 0.11
MH (mm)	4.81 ± 0.02	5.02 ± 0.22	4.82 ± 0.13	5.19 ± 0.16
MBD (mm)	2.25 ± 0.32	$1,99 \pm 0.21$	1.95 ± 0.12	1.86 ± 0.07
RH (mm)	12.08 ± 0.21	12.07 ± 0.30	$11.41 \pm 0.21^*$	$12.13 \pm 0.21\dagger$
MBH (mm)	5.24 ± 0.03	5.29 ± 0.04	5.22 ± 0.17	5.41 ± 0.09
GA (θ)	75.17 ± 1.57	76.73 ± 3.27	78.85 ± 0.90	79.18 ± 1.18
UIA (θ)	210.3 ± 8.63	210.2 ± 6.25	$283.4 \pm 28.3^*$	$249.9 \pm 19.7^*\dagger$
LIA (θ)	148.8 ± 6.01	147.1 ± 8.56	140.4 ± 4.91	134.2 ± 9.12

ML I, Mandibular length I; MBL, Mandibular body length; (ML II, Mandibular length II; MRCD, Mandibular ramus curvature depth; MH, Mandibular head; MBD, Mandibular base death; RH, Ramus height; MBH, Mandibular base height; GA, Gonial angle; UIA, Upper incisor angulation; LIA, Lower incisor angulation. Data are expressed as mean ± SEM from 10 animals per group. $^*p < .05$ from NG; $\dagger p < .05$ from RG.

($1.08 \pm 0.07\ mm^2$). In the mandibles from both irradiated rat groups inflammatory processes were found, but in RJ animals, the mandible showed a mild inflammatory process in comparison to RG animals.

Hematological parameters

Data related to hematologic parameters are given in Table 2. The total white blood cell count (WBC) was reduced in animals which received whole brain irradiation (WBI). RG animals showed decreased WBC by 47% in comparison to that in the NG control group. Black grape juice (BGJ) increased WBC by 118% when compared to that in the irradiated control group (RG). The red blood cell count (RBC) also decreased after irradiation. WBI animals showed reduced RBC by 9.2% in comparison to that in the NG control group. BGJ increased RBC by 8.3% when compared to that in the irradiated control group (RG). The hemoglobin content (HGB) detected in the irradiated control animals (RG) was 15% lower than the content found in non-irradiated animals (NG). Comparison between the irradiated group (RG) and irradiated, BGJ-supplemented group (RJ) showed an increase in HGB by 9.8% from RJ in relation to RG. Irradiation reduced hematocrit (HCT) by 14% in comparison to NG group. BGJ was able to increase HCT by 9.4% in the RJ group when compared to RG animals. Irradiation reduced the mean corpuscular hemoglobin concentration (MCHC) by 3.1% in comparison to the

Table 2. Hematological parameters from the experimental groups NG (non-irradiated; supplemented with glucose and fructose), NJ (non-irradiated; supplemented with black grape juice), RG (irradiated; supplemented with glucose and fructose) and RJ (irradiated; supplemented with black grape juice).

Parameter	NG	NJ	RG	RJ
WBC (10^9g/l)	7.13 ± 0.47	8.32 ± 0.73	3.80 ± 0.40*	8.29 ± 0.69†
RBC (10^{12}g/l)	8.46 ± 0.19	8.48 ± 0.08	7.68 ± 0.26 *	8.32 ± 0.16†
HGB (g/dl)	17.26 ± 0.35	16.78 ± 0.32	14.67 ± 0.30*	16.10 ± 0.37†
HCT (%)	45.20 ± 0.74	43.92 ± 0.81	38.60 ± 0.81*	42.22 ± 0.81*†
MCV (fl)	52.20 ± 0.58	52.33 ± 0.74	50.88 ± 0.43	51.00 ± 0.58
MCH (g)	19.84 ± 3.32	19.58 ± 0.31	19.40 ± 0.12	19.40 ± 0.19
MCHC (pg)	38.76 ± 0.25	38.20 ± 0.19	37.56 ± 0.15*	38.34 ± 0.14†
RDW (%)	16.66 ± 1.08	16.54 ± 1.19	17.37 ± 0.80	16.91 ± 0.65
PLT (10^9g/l)	558.50 ± 26.48	544.60 ± 34.49	480.55 ± 19.00	484.00 ± 44.36
PCT (%)	0.39 ± 0.02	0.39 ± 0.03	0.36 ± 0.01	0.39 ± 0.15
MPV (fl)	7.48 ± 0.31	7.18 ± 0.22	6.99 ± 0.34	7.16 ± 0.44
PDW (%)	28.35 ± 0.51	27.58 ± 0.39	27.21 ± 0.59	28.77 ± 0.95

WBC, White blood cell count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; PLT, platelet count; PCT, plateletcrit; MPV, mean platelet volume; PDW, platelet distribution width. Data are expressed as mean ± SEM from 10 animals per group. *$p < .05$ from NG; †$p < .05$ from RG.

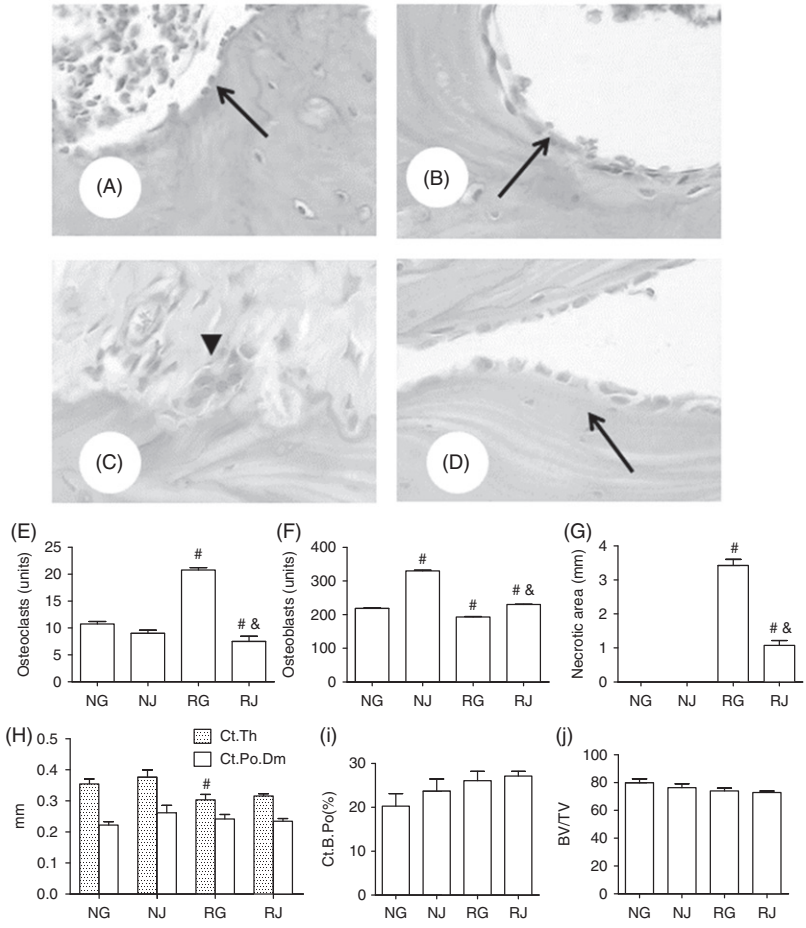

Figure 4. Mandibular bone sections (400× original magnification) from groups (A) NG (non-irradiated; supplemented with glucose and fructose), (B) NJ (non-irradiated; supplemented with black grape juice), (C) RG (irradiated; supplemented with glucose and fructose) and (D) RJ (irradiated; supplemented with black grape juice). Histomorphometric summary of (E) osteoclast count, (F) osteoblast count, (G) necrotic areas, (H) cortical thickness, cortical bone diameter (mm), (I) Cortical bone porosity (%), (J) Bone volume/tissue volume ratio (%). The arrows (→) indicate osteoblasts. The triangle (▼) indicates osteoclasts. Data are means ± SEM from 10 animals per group. #$p < .05$ from NG and $p < .05$ from RG.

concentration found in control animals (NG). BGJ was able to increase MCHC by 2.1% in relation to the RG group. The remaining hematological variables were not changed by radiation or BGJ supplementation.

Discussion

Black grape juice (BJG) has beneficial effects against damage induced by IR, such as hematopoietic syndrome (Ramos de Andrade et al. 2009), cardiac insults (de Freitas et al. 2013), spleen dysfunction (de Freitas et al. 2014) and cognitive impairments (Soares et al. 2014). In the present protocol, the amelioration of deleterious secondary effects of whole brain irradiation by BGJ was explored in rats, including anorexia, hematological changes and osteoradionecrosis.

Many protocols can be found in the literature related to radiomodifier administration, and some researchers have supplemented the animals only before and others only after irradiation. Without a consensus about the more adequate time to supplement the animals with radiomodifying substances, we opted for the following schedule: 4 days before X-rays exposure, during the X-rays exposure period, and 4 days after the last X-rays exposure (Figure 1). According to other authors the best time to initiate an antioxidant diet as countermeasure against radiation insults is 24 h after the irradiation, with a rat survival rate of about 80% (Brown et al. 2010). In the end, we decided to combine the supplementation procedure that had been previously used by our team with others described in the literature to achieve a greater life expectancy to irradiated animals (Guan et al. 2006; Ramos de Andrade et al. 2009; Brown et al. 2010; de Freitas et al. 2013, 2014).

Body weight and feeding after fractionated WBI

Most experimental studies using brain irradiation are focused on cognitive impairments, hippocampal neurogenesis and oxidative stress, but there are few data about systemic side effects caused by this modality of irradiation. However, clinical data have shown that some patients treated with radiotherapy for management of brain tumors can manifest anorexia and other systemic side-effects, even when the radiation dose delivered is localized and fractionated (Darzy 2009). Regarding feeding (Figure 2), food and water intake did not follow a standardized profile when compared to growing curves (Figure 1). Certainly, some rats may have been more affected by cranial irradiation or sham irradiation due to the wide variability found in rats from the same group. It was noticed that irradiated rats (RG) lost appetite during some days in the irradiation process (days 8, 9, 10, 13, 14, 15 and 16). Water intake also was affected. RG rats drank less water than control rats during the 3rd, 4th, 5th and 8th cranial irradiation sessions.

All in all, after irradiation or sham irradiation all rats experienced growth. However, approximately on day 61, both irradiated rat groups started losing weight dramatically. Although the animals were losing weight progressively, all appeared to be healthy. Closer inspection revealed that the upper incisors of the irradiated rats were abnormally long, restricting the gnawing activity. Our hypothesis was confirmed by morphometric measures realized on dissected mandibles followed by histopathologic analysis. All irradiated rats (RG and RJ groups) developed osteoradionecrosis (ORN), accompanied by enlargement of the upper incisors and under-growth of the lower incisors. This outcome can be explained considering that our fractionated irradiation protocol spared the frontal area, where the eruption zone of the upper incisors is located, but the mandible was affected. It has long been known that ionizing radiation produces a decrease in the growth of rat incisors (Kimeldorf et al. 1963; Ubios et al. 1992). The cranial fractionated irradiation window using cerrobend shields affected the growth rate of the lower incisors, leaving unaltered the upper ones. Albeit smaller, the length of the lower incisors in irradiated rats was not found to be significantly different from that of non-irradiated animals; however, the combination of decreased mandibular length and lower mandibular incisor growth could have conceivably resulted in an unimpeded incisor, a situation which altered the growth of the upper incisor, increasing its length (Burn-Murdoch 1995). Therefore, irradiated animals would have found it increasingly difficult to feed under these circumstances. Cranial irradiation has been repeatedly shown to induce body weight loss in the rat. Studies along 8–64 weeks have proved the progressive decrease in pituitary weight and function which can certainly explain this effect, but they have failed to show the possible reduction in feeding proficiency as a consequence of mandibular or maxillary alterations (Forbes et al. 2013). An experimental ORN rat model involving 50 Gy mandible irradiation reported slower body growth in animals euthanized at 10 weeks, consistent with our findings (Damek-Poprawa et al. 2013).

BJG supplementation and ORN

To date, there have been no studies investigating the mitigating effect of BGJ on mandible ORN caused by whole brain radiation (WBI). A diminution in these parameters is related to masticatory hypofunction (Guerreiro et al. 2013). Mandibular measurements revealed a positive effect of BGJ on mandibular length while decreasing upper incisor angulation (Table 1). Irradiated and BGJ-supplemented (RJ) rats were less affected by WBI than irradiated rats (RG), showing that BGJ supplementation improved the masticatory activity principally in the interval between 71 and 81 days (Figure 2(A)).

By histopathology analysis, we observed normal appearance in mandibular bones of control animals (Figure 4(A), (B)). The irradiated control group (RG) manifested a severe coagulation necrosis in the mandible, areas of devitalized bone, osteocytes with pyknotic and hyperchromatic nucleus, empty osteocyte lacunae (ghost cells), giant cells, increased osteoclastic activity and decreased osteoblastic activity (Figure 4(C)). These findings are in agreement with previous data (Cohen et al. 2011; Tamplen et al. 2011). Morphological measurements correlate with histopathological data, both showing a slight positive radiomodifying effect of BJG as

irradiated and BJG-supplemented (RJ) rats showed moderate necrosis, increased osteoblastic activity, a reduced number of osteoclasts (Figure 4(E)–4(G)) and a mild inflammatory process in mandibles (data not shown).

Black grape juice supplementation significantly increased osteoblastic activity in the control group (NJ), while cranial irradiation significantly increased osteoclasts in non-supplemented, irradiated (RG) animals. Taken together, the balance between radiation-induced osteoclastogenesis and grape juice-induced osteoblastogenesis could explain the lack of significant effect of grape juice supplementation in microCT morphometry parameters, cortical thickness, cortical pore diameter, and bone volume/total volume ratio. However, we found that cortical thickness was significantly affected by cranial irradiation, similarly to other reports (Kondo et al. 2009) in which bone volume fraction (BV/TV) was significantly reduced by irradiation as a result of increased porosity and decreased thickness; the trends shown in Figure 4(H, I, J) are consistent with this interpretation.

Medical treatment and prevention of ORN has been suggested to involve antioxidant supplementation with vitamin E, together with fibrosis-inhibiting agents (Lyons and Ghazali 2008; Fan et al. 2014). Our results contribute to reinforce the importance of antioxidant treatments to reduce the impact of this radiotherapy complication. The positive effect on body weight during the cranial irradiation observed in rats supplemented with BGJ can be associated with the presence of phenolic compounds in this beverage. In previous reports, our team quantified and isolated phenolic compounds of BGJ by high-performance liquid chromatography (HPLC), showing high levels of resveratrol and quercetin (de Freitas et al. 2013). Many polyphenols can cross the blood-brain barrier and increase the brain antioxidant activity (Youdim et al. 2004), as these molecules contain multiple hydroxyl groups active in radical scavenging and chelation (Heijnen et al. 2001; Kessler et al. 2003). Overall, the results suggest cumulative reactions to acute radiation on irradiated animals and an ameliorative effect in irradiated and BGJ-supplemented rats.

Flavonoids and other phenolic substances have been found to be bioactive compounds that improve bone mineralization (Yamaguchi et al. 2013). Caffeic acid, a compound present in BGJ, suppressed osteoclastogenesis on bone marrow in both in vitro and in vivo studies (de Freitas et al. 2013; Wu et al. 2012). *p*-hidroxycinnamic-derived phenolic acids such as cinnamic acid, ferulic acid, and caffeic acid stimulate osteoblastogenesis, which may induce differentiation of bone marrow mesenchymal stem cells, and suppress osteoclastogenesis (Yamaguchi 2013). The mechanism by which flavonoids and other phenolic compounds stimulate osteoblastic bone formation and attenuate osteoclastic bone resorption is believed to be mediated by suppression of NF-κB activation (Lai and Yamaguchi 2007). We can then assume that bioactive compounds in BGJ are able to attenuate the severity of ORN probably by improving osteoblastic activity and decreasing bone resorption as well as via anti-inflammatory action.

Hematologic syndrome has often been investigated in acute irradiation sickness (Blakely et al. 2007; Ramos de Andrade et al. 2009; Seed et al. 2014; Shao et al. 2014). Nonetheless, there is a lack of data related to hematologic parameters in whole brain irradiation (WBI) or hematologic syndrome related to ORN. This is the first report exploring the behavior of blood cells in a fractionated WBI experimental model. The changes observed in hematological parameters (Table 2) are consistent with an interpretation of systemic alterations occurring as a consequence of progressive decreased feeding activity in irradiated rats of the RG group and its improvement in BGJ-supplemented, irradiated rats (Figure 2).

Taken together, the results of the present study show a positive radiomodifying effect of black grape juice (BGJ) on systemic effects, such as alterations in food and water intake in some days of fractionated cranial irradiation. We can also conclude that BGJ can be useful to mitigate a severe complication of brain irradiation such as osteoradionecrosis (ORN) and the hematologic syndrome related to ORN. Nevertheless, BGJ supplementation did not completely restore the alterations induced by radiation on rats; the positive results collected will motivate further research.

Disclosure statement

The authors report no conflicts of interest. The authors alone are response for the content and writing of the paper.

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