Radioprotection of Sensitive Rat Tissues by Oligoelements Se, Zn, Mn Plus Lachesis Muta Venom

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Ionizing radiation/Radioprotection/Small intestine/Bone marrow/Oligoelements/Salivary glands.

In this study we first evaluated the general radioprotective efficacy of Se, Zn and Mn (4 μg/ml each) plus Lachesis muta venom (4 ng/ml) combination (O-LM) by determining survival on rats irradiated with lethal doses of gamma-rays. The aim of the second part of the study was to investigate the O-LM ability to prevent ionizing radiation-induced damage on small intestine, bone marrow and submandibular glands. Hence, histological characteristics and functional studies, together with proliferation and apoptotic marker levels on whole body irradiated rats with a 5 Gy dose were evaluated. Results show that all animals of the untreated group died after whole body irradiation with 8 and 10 Gy while 60 day-survival was more than 80% and 40% in O-LM-treated animals, respectively. Histopathological examinations revealed a high degree of small intestine and submandibular gland radioprotection 3 days post-irradiation. O-LM inhibited histological damage on small intestine, restoring the radiation-induced reduction in villous height and crypt number. O-LM prevented radiation-induced loss of salivary gland function and morphological alterations. These effects were associated to a complete inhibition of radiation-induced apoptosis. Furthermore, studies performed 30 days post-irradiation revealed that O-LM significantly improved bone marrow repopulation, increasing all medullar progenies to the extent of the non-irradiated animals, and completely prevented permanent submandibular gland alterations. Based on the present results and taking into account that O-LM is being safely administered in phase I clinical trial as an immunomodulator, we conclude that O-LM is a non-toxic promising approach to achieve radioprotection for patients undergoing radiotherapy.

INTRODUCTION

Radiotherapy is one of the most widely used local modality for the treatment of cancer. Despite its high therapeutic index defined as the ratio of tumour response to normal-tissue damage, radiotherapy can cause disabling normal tissue injury causing serious adverse effects to patients.[1,2] Therefore, a great challenge of modern radiotherapy is to increase the tolerance of normal tissues by using radiopro-tectors to improve patient quality of life. The search for ideal protective agents for use in a variety of radiation scenarios has continued for more than six decades. Surprisingly, few radiation protectors are in use today and, furthermore, their clinical use is limited due to their toxicity. Therefore, a more global view of the pathogenic process induced by ionizing radiation is required to develop effective and nontoxic agents to protect normal tissues from radiation injury.[2–4]

Actively proliferating and undifferentiated cells are the most highly sensitive to radiation and include the stem cells of the self-renewing systems such as intestinal crypt cells and bone marrow stem cells.[1,5] Thus, during radiotherapy for intraabdominal and pelvic cancers, the small intestine and bone marrow are seriously affected by ionizing radiation.[1,6]

Although salivary glands should be considered to be radioresistant because of their highly differentiated cellular state, salivary acinar cells are exquisitely sensitive to radiation, and xerostomia is an early and persistent sequel of head and neck irradiation.[7]

We have previously demonstrated the protective effect of

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a novel combination composed of three oligoelements [Selenium (Se), Zinc (Zn), Manganese (Mn)] and Lachesis muta venom (O-LM) against carcinogenic drugs and high doses of chemotherapy.[8] Recently, we have shown that O-LM treatment significantly protected mouse small intestine and bone marrow against ionizing radiation toxicity.[9]

In order to evaluate the general radioprotective efficacy of O-LM, rats were irradiated with gamma-rays and their survival (8 and 10 Gy) and body mass gain (2 and 5 Gy) were monitored during a 60 day follow up period. The aim of the second part of the study was to investigate the ability of O-LM to prevent ionizing radiation-induced damage on small intestine, bone marrow and submandibular glands of rats irradiated with a dose of 5 Gy. For that purpose, the histological characteristics of these tissues and proliferation and apoptotic marker levels in whole body irradiated-rats were evaluated.

MATERIALS AND METHODS

Treatment and irradiation

In all studies the treatment employed (O-LM) was a solution composed of a combination of the oligoelements Zn, Se, and Mn (4 μg/ml each) (Merck, Argentina) plus Lachesis muta venom (LM) (4 ng/ml) (Iquitos, Departamento de Loreto or Cenepa, Mamayaque, Peru). The LM dose used in our experiment is about 1000 times lower than the 50 lethal dose (4.7 mg/kg body weight).[10]

A ^{137}Cs source of 189 TBq (7 Gy/min) calibrated by Argentine National Commission of Atomic Energy with a TLD 700 dosimeter and validated by Argentine Nuclear Regulatory Authority was employed.

Male Sprague-Dawley rats, weighing 200–230 g were purchased from the Division of Laboratory Animal Production, School of Veterinary Sciences, University of La Plata, Buenos Aires and were randomly separated into different groups. Rats were maintained in our animal health care facility at 22 to 24°C and 50% to 60% humidity on a 12 h light/dark cycle with food and water available *ad libitum*.

Animals were randomly separated into 4 groups: group 1, non-irradiated rats receiving daily subcutaneous (sc) saline; group 2, whole-body irradiated rats with a single dose ranging from 2 to 15 Gy receiving daily sc saline; group 3, non-irradiated rats receiving 0.5 ml daily sc O-LM treatment starting 30 days before irradiation; group 4, whole-body irradiated rats with a single dose ranging from 2 to 15 Gy receiving 0.5 ml daily sc O-LM treatment starting 30 days before irradiation.

Parameters recorded were: 50 lethal dose at 30 days after whole body irradiation (^{30}LD$_{50}$) value, survival and body weight that were monitored during the period of 60 days after irradiation according to the experimental design described in Table 1. In all deceased or sacrificed animals necropsy and histopathological examination were performed.

In addition, to determine the potential radioprotective effect of O-LM on rat small intestine, bone marrow and submandibular glands we used the same experimental procedures that we previously described in detail to evaluate the radioprotective effect on mouse small intestine and bone marrow.[9] Briefly, 3 or 30 days post 5 Gy whole-body irradiation metacholine-induced salivary secretion was measured or animals were sacrificed by cervical dislocation and salivary glands, bone marrow, and small intestines were removed, fixed, and histological and histochemical characteristics were evaluated, as indicated in Table 2.

Animal procedures were in accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Research Council, USA, 1996, and protocols were approved by the Ethical and Educational Committee of the Institute of Immunooncology.

Salivary secretion

Salivation was assessed in anesthetized rats (chloralose 100 mg/kg, 0.5 ml NaCl-0.9%) (FLUKA, Berlin, Germany). The right femoral vein was cannulated with a polyethylene catheter (P40 catheter, Rivera & Co, Argentina) to administer the sialagogic agonist, metacholine. Through a midline incision in the neck the trachea was intubated and the submandibular gland ducts were exposed and cannulated with a fine glass cannula to collect saliva samples. No basal salivation was observed from the glands. Salivation was induced by the administration of different concentrations of metacholine ranging from the threshold dose to that exerting the maximum stimulus (0.3, 1, 3, 10 and 30 μg/kg, in saline) (FLUKA) sequentially injected via the right femoral vein. Salivary samples were collected for 3 minutes in pre-

Table 1. Experimental design for ^{30}DL$_{50}$, 60 day survival and body weight determinations

Group	Description	*s.c.* injection (0.5 ml/day)	N	Doses (Gy)
1	Untreated	saline	8	—
2	Irradiated	saline	48 (8 per dose)	2, 5, 8, 10, 12, 15
3	O-LM treated	O-LM*	8	—
4	O-LM treated and irradiated	O-LM*	48 (8 per dose)	2, 5, 8, 10, 12, 15

*O-LM treatment started 30 days before irradiation and continued during the 60 day follow-up period.

Table 2. Experimental design for histopathological and functional studies

Group	Description	*s.c.* injection (0.5 ml/day)	N[#]	Doses (Gy)
1	Untreated (3rd day)	saline	14	—
	Untreated (30th day)	saline	14	—
2	Irradiated (3rd day)	saline	14	5
	Irradiated (30th day)	saline	14	5
3	O-LM treated (3rd day)	O-LM*	14	—
	O-LM treated (30th day)	O-LM*	14	—
4	O-LM treated and irradiated (3rd day)	O-LM*	14	5
	O-LM treated and irradiated (30th day)	O-LM*	14	5

*O-LM treatment started 30 days before irradiation and continued during the follow-up period (3 or 30 days post irradiation). [#]6 and 8 animals were used to evaluate salivary secretion and histopathological studies, respectively.

weighed aluminum foil and the quantity of saliva was determined by weighing, as previously described.[11] Three additional minutes were allowed until the administration of the next dose. Results were expressed as mg of saliva per gland.

Histopathological studies

The small intestine was opened along the mesenteric border and samples were pinned flat on cork board, mucosal side up, to allow immediate fixation with 10% neutral buffered formalin and comparable samples. Bone marrows were fixed with Bouin's solution while salivary glands were fixed with 10% neutral buffered formalin. Tissue samples were embedded in paraffin and cut into 3 μm thick serial sections. Tissue morphology was examined on tissue sections after hematoxylin-eosin staining as we have previously described.[9]

Immunohistochemical staining

After deparaffinization, specimens were placed in citrate buffer (10 mM, pH 6.0) and heated in a microwave oven twice for 2 minutes at boiling temperature for antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distillated water. After blocking, slides were incubated with primary mouse anti PCNA (1:100, DakoCytomation, Glostrup, Denmark) antibody overnight in a humidified chamber at 4°C. Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-mouse and developed by diamino-benzidine staining (Sigma Chemical Co., St. Louis, MO, USA). Slices were counterstained with hematoxylin and light microscopy was performed on an Axiolab Karl Zeiss microscope (Göttingen, Germany). All photographs were taken using a Canon PowerShot G5 camera (Tokyo, Japan). The immunostaining assessment was performed blind to the data in all tests. To control the signal specificity, serial sections were made from five selected positive cases which were subjected to the same staining procedure, with either a normal mouse or phosphate buffered saline (PBS) to replace the first antibody. This control staining did not give rise to a signal. Determinations were made in cells of crypts and villi and submandibular glands and at least 10 fields were examined.

Determination of apoptosis

Apoptotic cells were determined by terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) assay using ApoptagTM plus peroxidase in situ apoptosis Detection Kit (CHEMICON International, Temecula, CA, USA) according to the manufacturer's instructions. Tissues were visualized using Axiolab Karl Zeiss microscope (Göttingen, Germany). All photographs were taken using a Canon PowerShot G5 camera (Tokyo, Japan). Results were expressed as the number of TUNEL-positive cells per crypt for the small intestine and as the number of TUNEL-positive cells per field in the submandibular gland, and at least 10 fields were examined.

Statistical analysis

All statistical analyses were performed with GraphPad Prism Version 5.00 software (San Diego, CA, USA) as it is described in each case. P values < 0.05 were considered significant.

RESULTS

O-LM increases survival after whole body irradiation

In order to investigate the global protective effect of O-LM, we first evaluated the survival monitored during a 60 day follow up period after whole body irradiation with different doses of ionizing radiation. Results indicate that O-LM treated animals exhibited a higher survival rate compared to the untreated ones after whole body irradiation
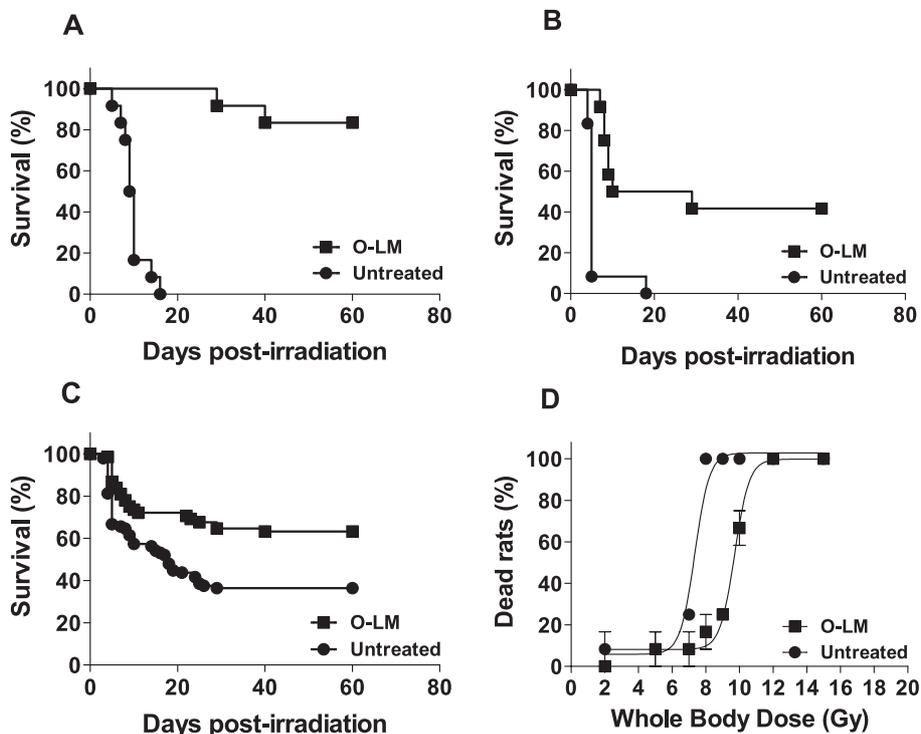
Fig. 1. Evaluation of survival. Kaplan Meier survival curves **A)** after 8 Gy dose (P < 0.0001, Log rank test and Gehan-Breslow-wilcoxon test), **B)** after 10 Gy dose (P < 0.001, Log rank test and Gehan-Breslow-wilcoxon test), and **C)** considering all batches of treated and irradiated versus untreated and irradiated rats (P < 0.0005, Log rank test and Gehan-Breslow-Wilcoxon test). **D)** $^{30}LD_{50}$. O-LM treatment significantly increases $^{30}LD_{50}$ (P < 0.01, T-test).

with doses of 8 Gy (83% vs. 0%) and 10 Gy (42% vs. 0%) doses (Fig. 1A, 1B). Similarly, a noticeable difference was observed between the global survival of all batches of O-LM treated and irradiated versus untreated and irradiated rats (Fig. 1C). In agreement with these results, $^{30}LD_{50}$ was markedly higher in O-LM treated animals (9.7 ± 0.1 Gy vs. 7.4 ± 0.1 Gy, P < 0.01, T-test), showing a wright shifting to the right of the O-LM $^{30}LD_{50}$ curve and therefore, clearly demonstrating the radioprotective effect of O-LM (Fig. 1D).

Furthermore, rats irradiated with a dose of 5 Gy receiving O-LM showed no significant modification of body weight, demonstrating a better preservation, and also a faster recovery of body weight than control untreated animals (Fig. 2).

O-LM prevents ionizing radiation-induced damage on small intestine

Histopathological studies performed 3 days post irradiation indicate that ionizing radiation significantly altered small intestine mucosa, characterized by a decreased number of crypts, anisokaryosis, ulceration and villous edema. O-LM treatment reduced mucosal atrophy after irradiation, increasing the number of crypts per circumference (187.3 ± 3.8 vs. 149.8 ± 9.6 in untreated and irradiated rats) while reducing edema and ulceration. It is interesting to point out that O-LM administration did not produce histological mod-
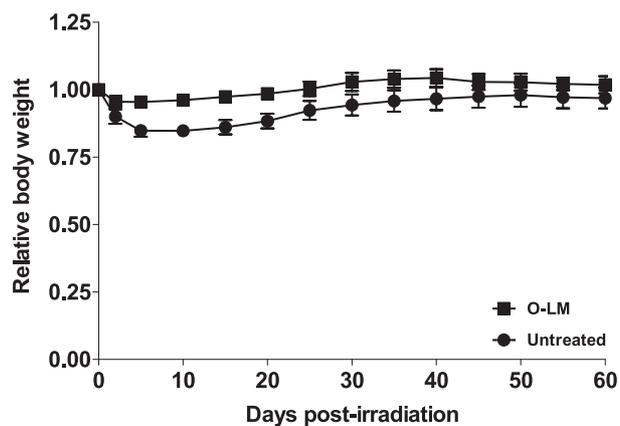

Fig. 2. Chronologic evolution of relative body weight of 5 Gy irradiated, treated and untreated rats. Better preservation and recovery is observed in treated compared to untreated rats.

ifications in non-irradiated animals (Fig. 3, Table 3).

To investigate whether O-LM could be modulating cell proliferation and apoptosis, we next evaluated proliferation and apoptotic markers in this tissue. With regard to proliferation, results show that PCNA expression was high in crypts not only in non-irradiated but also in irradiated groups while O-LM did not significantly affect its expression, suggesting
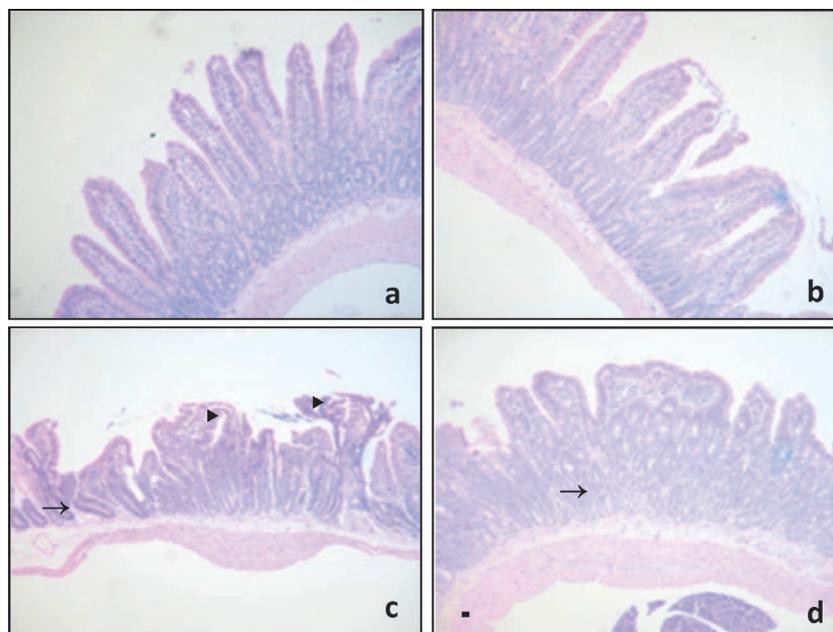
Fig. 3. Effect of O-LM on the small intestine mucosal damage exerted by ionizing radiation. **a)** Normal appearance of untreated and **b)** O-LM treated rats small bowels. **c)** Crypt number reduction (arrows), ulceration and villous edema (arrow heads) observed in irradiated rats. **d)** Partial preservation of the crypts (arrows), absence of edema and ulcerated sectors in small bowel of irradiated and O-LM treated rats. Pictures were taken at 100× magnification. Scale bar = 20 μm.

Table 3. Histological characteristics of rat small intestine

Group	Mucosal trophism	crypts/Circumference[¶]	Mucosal ulceration	Others changes
Untreated[a]	Normal	215.3 ± 13.0	Absent	–
O-LM[b]	Normal	198.0 ± 5.1	Absent	–
Untreated-5 Gy[c]	Marked atrophy	$149.8 \pm 9.6*$	Severe	Anisokariosis/villous edema/ vascular damage
O-LM-5 Gy[d]	Mild atrophy	$187.3 \pm 3.8\#$	Mild	Slight villous edema

[¶]Mean value of the experimental group calculated from the average number of crypts of 10 specimens examined. [a]Representative of small intestines from at least six saline-treated rats. [b]Representative of small intestines from at least six daily O-LM-treated rats. [c]Representative of small intestines from at least six saline-treated and 5 Gy-irradiated rats. [d]Representative of small intestines from at least six daily O-LM-treated and 5 Gy-irradiated rats. *$P < 0.01$ and #$P < 0.01$ compared with the untreated and the untreated-5 Gy groups, respectively (ANOVA and Newman-Keuls Multiple Comparison Test).

that 3 days after a 5 Gy radiation dose intestinal crypts are in regeneration (Fig. 4).

The protective effect of O-LM on small intestine was associated to a reduction of ionizing radiation-induced apoptosis determined by the TUNEL assay. In non-irradiated rats, the number of apoptotic cells in crypts was low and was not modified by O-LM treatment. On the contrary, ionizing radiation notably increased the number of apoptotic cells per crypt, while O-LM treatment reversed completely this effect (Fig. 5, Table 4).

O-LM enhances bone marrow repopulation after whole body irradiation

We also explored the effect of O-LM on other radiosensitive tissue like bone marrow. Results indicate that ionizing radiation exposure produced a total aplasia (grade III) after 3 days post irradiation, and O-LM treatment did not significantly prevent this effect (data not shown). However, histopathological studies performed 30 days post irradiation revealed that O-LM daily administration significantly improved bone marrow repopulation after irradiation, increasing all medullar progenies to the extent
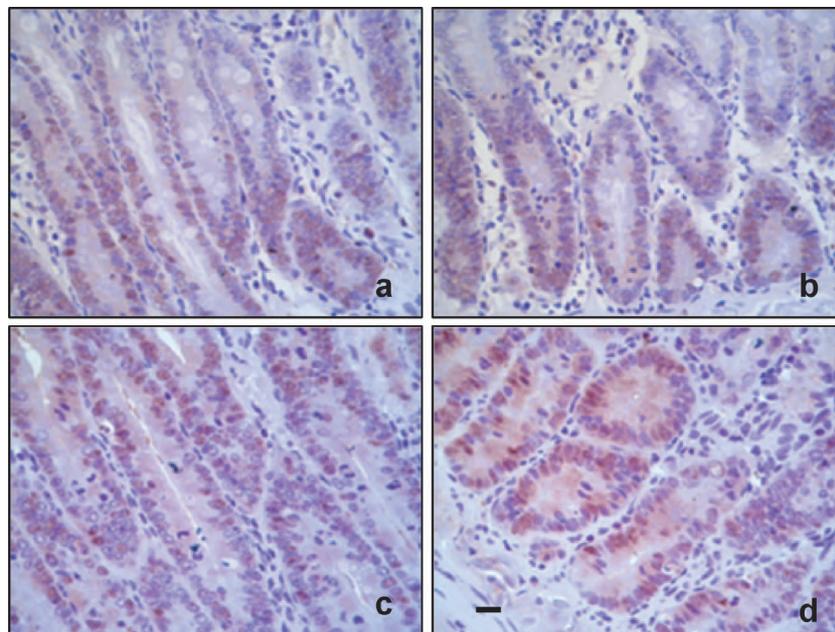
Fig. 4. Effect of O-LM treatment on PCNA immunoreactivity in small intestine. Similar PCNA immunore-activity was observed in **a)** untreated, **b)** O-LM treated, **c)** untreated and irradiated, and **d)** O-LM treated and irradiated intestinal crypts. Pictures were taken at 630× magnification. Scale bar = 20 μm.
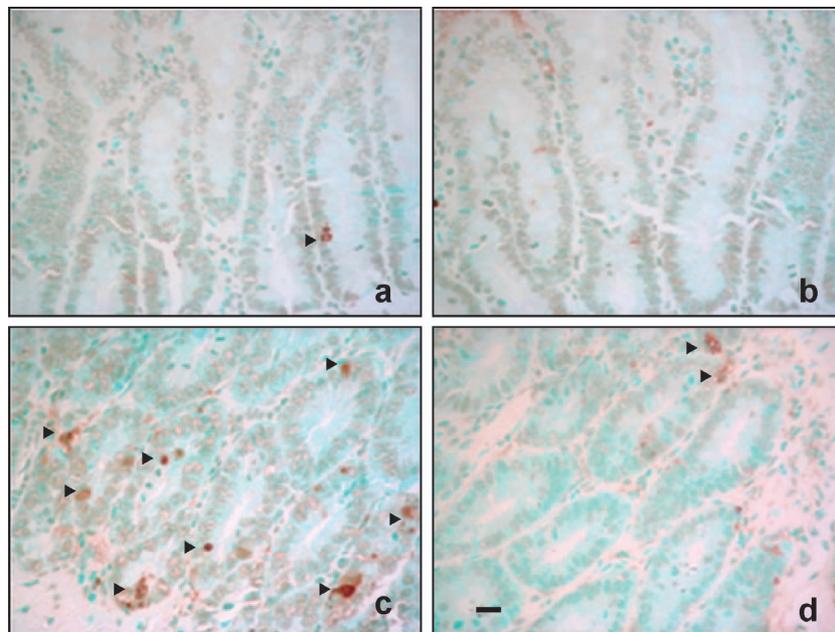

Fig. 5. Effect of O-LM treatment on cell apoptosis in small intestine. Absence or low number of apoptotic cells in **a)** untreated and **b)** O-LM treated rats. **c)** Crypts of irradiated rats showing frequent apoptotic cells (arrow heads). **d)** Intestinal crypts of irradiated and O-LM treated rats showing very scarce apoptotic cells. Pictures were taken at 630× magnification. Scale bar = 20 μm.

of the non-irradiated animals, compared to a grade I of bone marrow aplasia in the untreated animals. In non-irradiated rats, O-LM did not affect bone marrow trophism (Fig. 6, Table 5).

O-LM preserves morphology and function of irradiated rat submandibular gland

Functional and structural changes have been observed in salivary glands after ionizing radiation exposure.[12,13] There-fore, we additionally studied whether O-LM has the ability

to protect submandibular gland against ionizing radiation damage. Results demonstrate that 3 days after exposure, ionizing radiation reduced salivary secretion between 38 and 58% at 10 and 30 μg/kg of metacholine dose, respectively.

Table 4. Number of apoptotic cells in small intestinal crypts. See key for groups in Table 3.

Untreated[a]	O-LM[b]	Untreated-5 Gy[c]	O-LM-5 Gy[d]
0.2 ± 0.1	0.2 ± 0.1	1.5 ± 0.3*	0.4 ± 0.2#

Apoptotic cells were determined using TUNEL assay. The number of TUNEL positive cells per crypt is expressed as mean ± SEM. *$P < 0.001$ compared with the untreated group; #$P < 0.01$ compared with the untreated-5 Gy group (ANOVA and Newman-Keuls Multiple Comparison Test).

Additionally, radiation produced severe changes in gland structure that include reduced height of granular convoluted ducts and vacuolization. O-LM administration remarkably preserved gland function, completely reversing the inhibitory effect on salivary secretion produced by ionizing radiation. Moreover, O-LM prevented the histological damage on submandibular gland induced by ionizing radiation (Fig. 7A, 7B). No effects were observed in non-irradiated animals by O-LM administration (Fig. 7A, 7B). The radioprotective effect of O-LM was related to a marked decrease in gland apoptosis induced by radiation. In non-irradiated animals, the number of apoptotic cells in submandibular gland was low and it was not affected by O-LM treatment. Conversely, ionizing radiation significantly increased apoptosis in acinar and ductal cells. Interestingly, O-LM treatment completely
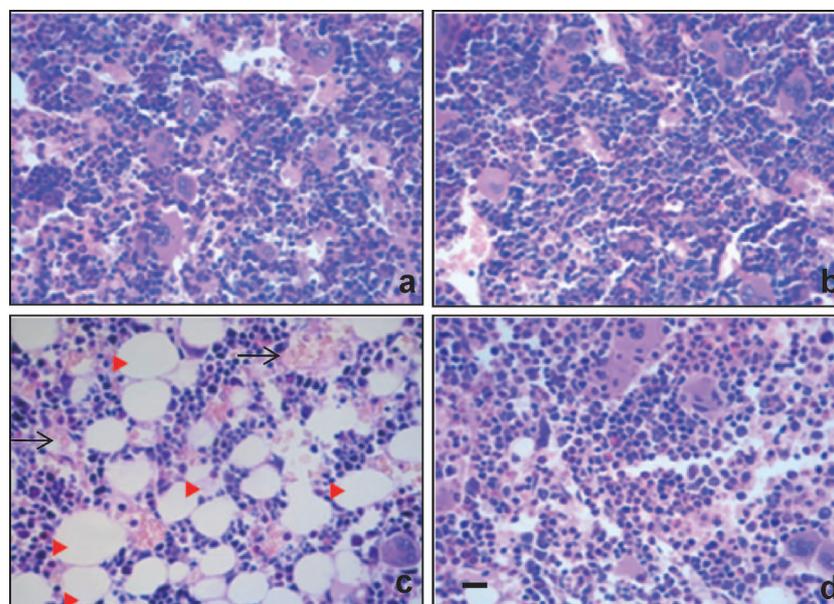

Fig. 6. Effect of O-LM on bone marrow repopulation evaluated 30 days after whole body irradiation. Normal trophism of **a)** untreated and **b)** O-LM-treated bone marrow. **c)** Bone marrow of irradiated rats, showing mild adipose replacement (arrow heads) of the normal cytological bone marrow population and congestive blood vessels (arrows). **d)** Bone marrow of O-LM-treated and irradiated animals demonstrating significant preservation of bone marrow. Pictures were taken at 630× magnification. Scale bar = 20 μm.

Table 5. Histological characteristics of rat bone marrow. See key for groups in Table 3.

Group	Trophism	Myeloid cells per mm²¶	Lymphoid cells per mm²¶	Megakaryocytes cells per mm²¶	Erythroidcells per mm²¶	Stromal changes
Untreated[a]	Normal	400 ± 35.5	107.6 ± 14.5	16.1 ± 2.1	291.4 ± 50.5	Without alterations
O-LM[b]	Normal	425 ± 25.0	110.0 ± 10.0	17.0 ± 2.0	325.0 ± 25.3	Without alterations
Untreated-5 Gy[c]	Grade I Aplasia	180.3 ± 39.7*	75.4 ± 3.9*	5.2 ± 2.6*	178.3 ± 23.4*	Congestive vessels, microhemorrhage
O-LM-5 Gy[d]	Normal	374.2 ± 10.5#	111.7 ± 17.3#	13.9 ± 3.1#	254.0 ± 30.4#	Moderate vascular congestion

O-LM preserves histopathological characteristics of rat bone marrow after ionizing radiation exposure. ¶Mean value of the experimental group calculated from the average number of cells per square millimeter (mm²) of bone marrow. Error bars represent the means ± SEM. *$P < 0.01$ vs. untreated, #$P < 0.01$ vs. untreated-5Gy (ANOVA and Newman-Keuls Multiple Comparison Test).
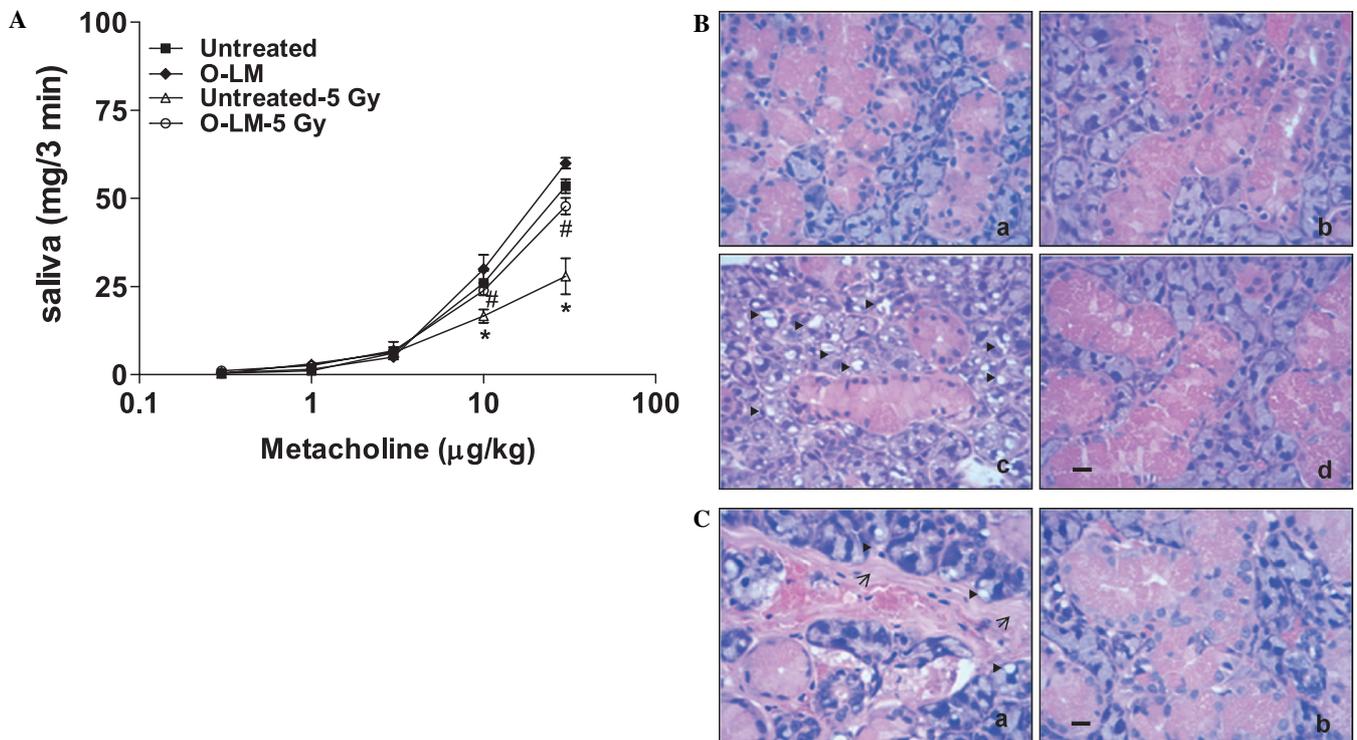
Fig. 7. **A)** Effect of O-LM on submandibular gland functionality 3 days post irradiation. Mean salivary secretion in irradiated and non-irradiated, untreated and O-LM-treated rats. Error bars represent the mean ± SEM. *P < 0.01 vs. untreated, #P < 0.01 vs. untreated-5 Gy (ANOVA and Newman-Keuls Multiple Comparison Test). **B)** Histopathological study of submandibular gland 3 days post irradiation. Normal appearance of submandibular gland from **a)** untreated and **b)** O-LM treated rats. **c)** Submandibular gland from irradiated rats, demonstrating structural changes and vacuolization (arrow heads). **d)** Submandibular gland from irradiated and O-LM treated rats, exhibiting preserved gland structure. **C)** Histopathological study of submandibular gland 30 days post irradiation. **a)** Submandibular gland from irradiated rats, showing altered epithelial organization, fibrosis (arrows) and vacuolization (arrow heads). **b)** Submandibular gland from irradiated and O-LM treated rats, demonstrating normal appearance of gland structure. Pictures were taken at 630× magnification. Scale bar 20 μm.
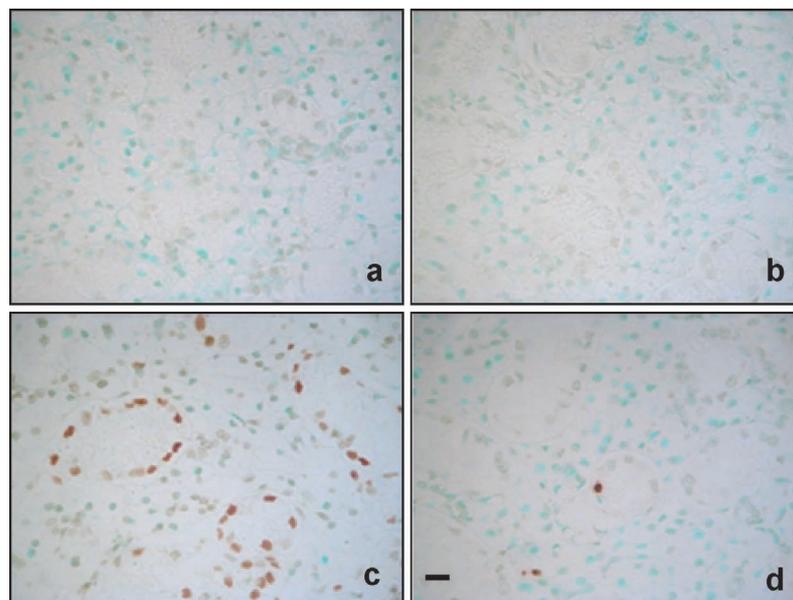

Fig. 8. Effect of O-LM in submandibular gland apoptosis. Almost absence of apoptotic cells in submandibular gland of **a)** untreated and **b)** O-LM treated animals. **c)** Massive presence of apoptotic cells in ductal and acinar cells of irradiated submandibular gland. **d)** Outstanding reduction of apoptosis in submandibular gland of irradiated and O-LM treated rats. Pictures were taken at 630× magnification. Scale bar 20 μm.

Table 6. Effect of O-LM on cell proliferation and apoptosis of submandibular gland. See key for groups in Table 3.

Group	TUNEL-positive cells/field[¶]	PCNA-positive cells/field[¶]
Untreated[a]	0.1 ± 0.1	4.2 ± 0.8
O-LM[b]	0.2 ± 0.1	3.7 ± 0.4
Untreated-5 Gy[c]	47.5 ± 2.2*	0.2 ± 0.1*
O-LM-5 Gy[d]	1.4 ± 0.2#	0.3 ± 0.1*

[¶]The number of TUNEL and PCNA-positive cells per field are expressed as mean ± SEM. *P < 0.001 compared with the untreated group; #P < 0.001 compared with the untreated-5 Gy group (ANOVA and Newman-Keuls Multiple Comparison Test).

Table 7. Effect of O-LM on salivary secretion 30 days post radiation exposure. See key for groups in Table 3.

Group	MC (10 µg/Kg)[¶]	MC (30 µg/Kg)[¶]
Untreated[a]	34.8 ± 1.0	56.0 ± 1.0
O-LM[b]	36.0 ± 1.5	60.1 ± 3.2
Untreated-5 Gy[c]	11.3 ± 1.3*	28.0 ± 2.0*
O-LM-5 Gy[d]	32.0 ± 0.5#	71.8 ± 0.8*#

[¶]Salivation was stimulated with metacholine (MC) and results are expressed as mean of the mg of saliva ± SEM. *P < 0.01 compared with the untreated group; #P < 0.01 compared with the untreated-5 Gy group (ANOVA and Newman-Keuls Multiple Comparison Test).

inhibited the ionizing radiation induced apoptosis in submandibular gland (Fig. 8, Table 6). Regarding the proliferation marker, ionizing radiation significantly decreased PCNA expression in salivary gland and O-LM was not able to prevent this effect (Table 6). In non-irradiated animals, O-LM did not alter either apoptosis or proliferation of submandibular gland (Fig. 8, Table 6).

We also evaluated whether O-LM treatment could have a prolonged radioprotective effect on salivary gland by studying its action after 30 days post irradiation. Our findings show that ionizing radiation produced a permanent alteration in submandibular gland functionality, reducing salivary secretion stimulated with 10 and 30 µg/kg of metacholine dose by 50–70%, which was associated to an alteration in gland structure with fibrosis and vacuolization (Fig. 7C, Table 7). O-LM treatment in irradiated animals completely prevented the reduced salivation induced by radiation while it preserved submandibular gland morphology (Fig. 7C, Table 7).

DISCUSSION

Cancer is one of the principal causes of morbidity and mortality worldwide, and radiotherapy is a leading and effective cancer treatment modality. About half of all cancer patients receive some type of radiation therapy sometime during the course of their treatment.[14] Unfortunately, radiation can cause unwanted side effects on normal tissues, resulting from the depletion of a cell population by cell killing.[1] Consequently, there is a need to further understand the mechanisms of radiation damage and to develop an effective and non-toxic radioprotective drug.[1,3,14–17]

In the present study, we aimed to demonstrate the prevention of radiation-induced damage produced by O-LM in an experimental rat model. In order to evaluate the general radioprotective efficacy of O-LM, rats were irradiated with a lethal dose of gamma-rays and their survival was monitored during a 60 day period after irradiation. Furthermore, body weight gain after a sublethal dose was evaluated. Results show that all animals from the untreated group died after whole body irradiation while more than 80% and 40% of the O-LM-treated animals survived the lethal dose of 8 and 10 Gy, respectively, demonstrating that O-LM remarkably improved the 60-day survival of irradiated rats. Accordingly, O-LM administration increased the $^{30}LD_{50}$ by 31% and a similar LD_{50} evaluated 60 days post irradiation was obtained (data not shown). In addition, the decrease in body weight was almost completely prevented and a faster recovery by O-LM treatment was observed. Animal necropsy revealed that anatomopathological alterations, principally in bone marrow (Grade III aplasia), small intestine severe ulceration and opportunistic bacterial and fungal infections were also prevented in irradiated and treated rats (data not shown). These results explain the better survival rates observed in the treated group at the different irradiation doses.

It is well established that during radiotherapy delivered to pelvis and abdomen, ionizing radiation significantly injured highly radiosensitive tissues such as small intestine and bone marrow.[1,18,19] Furthermore, salivary glands are the most affected organs during radiotherapy of the head and neck region.[13,20,21]

Therefore, we next investigated the tissue-protective effects of O-LM on rats irradiated with a dose of 5 Gy. Histopathological examinations revealed a high degree of radioprotection of small intestine and submandibular gland 3 days post-irradiation. O-LM inhibited histological damage on small intestine, restoring the radiation-induced reduction in villous height and crypt number, and therefore conserving the mucosal trophism. In line with these observations, O-LM completely prevented radiation-induced apoptosis in intestinal crypts, indicating a protective effect on gastrointestinal tract.

Furthermore, O-LM inhibited radiation-induced loss of salivary gland function and damage to the gland architecture, including vacuolization and alteration of granular convoluted ducts. In agreement with results in small intestine, O-LM completely reversed radiation-induced apoptosis in acini and ducts of submandibular gland. In both tissues, O-

LM was not able to counteract the radiation-induced decrease in proliferation.

Results demonstrate that a total bone marrow aplasia (grade III) was observed 3 days post irradiation. O-LM treatment did not significantly prevent this deleterious effect (data not shown). However, histopathological studies performed 30 days post irradiation revealed that O-LM administration significantly improved bone marrow repopulation after irradiation. Influence of ionizing radiation and O-LM administration on submandibular gland was also evaluated 30 days after irradiation. Our findings show that O-LM exhibited a prolonged radioprotective effect on salivary gland, completely inhibiting the functional and morphological gland impairment.

It is important to point out that O-LM achieved an almost total protection of the studied tissues, reaching the functional and morphological characteristics of the non-irradiated tissues. Accordingly, we have previously reported in other rodent specie that O-LM treatment effectively mitigated the ionizing radiation induced toxicity on mouse small intestine and bone marrow.[9] In addition, we determined that O-LM protected BALB/c mice from irradiation by recovering the immune function, improving T lymphocyte activity and modulating the production of key cytokines as IFNγ and TNFα (Crescenti *et al.*, unpublished). Lachesis muta venom consists of a great variety of protein including A2 phospholipase, metalloproteinases, and serine proteases.[22] It was also reported that lysophosphatidylcholine produced by the phospholipase A2 isolated from Lachesis muta snake venom stimulated natural killer activity.[23]

In line with these observations, reports from different animal experiments indicate that antioxidant nutrients, such as selenium compounds, are protective against lethality and other radiation effects but to a lesser degree than most synthetic protectors.[24] Selenium has been shown to have a radioprotective action in rat intestines[25] and to increase survival in animals.[21,26] Accordingly, various studies described a cytoprotective effect on the acute toxicity of the salivary glands of rats during irradiation with synchronous application of sodium selenite although they assessed higher concentrations than the one used in the present study.[21,27,28] Furthermore, the significant benefits of sodium selenite supplementation with regards to radiotherapy induced diarrhea in patients with cervical and uterine cancer has been demonstrated in a prospective randomized trial.[29,30]

Selenium and other essential trace elements such as Zn and Mn, are crucial cofactors in the most important endogenous antioxidative systems of the human body.[26] Mn and Zn complexes have also been found to prevent death in lethally irradiated mice. These pharmacological effects of essential metalloelement chelates can be understood as due to facilitation of de novo synthesis of essential metalloelement-dependent enzymes required to repair radiation injury.[31,32]

Our findings clearly confirmed that O-LM exhibits an important protective effect against whole-body gamma-radiation and the inhibition of apoptosis is the probable mechanism of action involved.

Due to the increased use of ionizing radiation in various aspects of human life, especially in areas pertaining to radiotherapy of cancer, food preservation, agriculture, industry and power generation, there is a need to develop an effective and non-toxic radioprotector.[14,17] O-LM demonstrated a total absence of toxicological or undesirable side effects in two rodent and one non-rodent species, both in acute and chronic studies. Furthermore, O-LM has been administered in a completely safe way to patients in phase I clinical trial as an immunomodulator (Reference: National Administration of Food, Drugs and Medical Technology of Argentina–ANMAT-1-47-6840-08-4). The protocol involved the intravenous administration of 3 different doses of O-LM (50, 100, 150 ml per day) to healthy volunteers during 30 days. No adverse effects were observed during the 30 day administration or observation periods at any dose.

It is interesting to highlight that O-LM treatment was also able to radiosensitize breast malignant cells.[9]

Therefore, we conclude that the novel non-toxic combination of O-LM is a valuable and promising approach to achieve radioprotection and recovery from radiation-induced injury experienced by patients undergoing radiotherapy for cancer treatment and also by individuals who may suffer environmental, occupational, or accidental exposure to ionizing radiation.

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