# Oligoelements Se, Zn, Mn Plus Lachesis Muta as Radioprotectors of Normal Tissues and Radiosensitizers of Malignant Cells

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**Abstract.** The *in vitro* effect of O-LM on radiosensitivity of different human cell lines and the *in vivo* tolerance to high doses of ionizing radiation induced by the combination of Se, Zn and Mn plus Lachesis *muta* (O-LM) was evaluated. Sprague-Dawley rats received whole-body irradiation with a single dose (2 to 15 Gy) employing a $^{137}Cs$ source of 189 TBq (7.7 Gy/min). A half of rats received daily sc O-LM starting 10 days before irradiation. These animals receiving 8, 10, and 12 Gy showed significantly higher survival versus controls ($p<0.0001$, 0.003 and 0.0083, respectively). $^{30}LD_{50}$ value for O-LM treated rats was 9.6 Gy vs. 5.8 Gy for controls ($p<0.0001$). The protective effect of O-LM was also *in vivo* evaluated on small intestine and bone marrow of nude mice irradiated with a single whole-body dose of 10 Gy. Mice were sacrificed 5 days after irradiation. Mice receiving sc daily O-LM starting 90 days before irradiation, showed better villous and crypts conservation, lack of oedema and vascular damage in comparison to controls. Bone marrows of treated animals showed grade I-II aplasia instead of grade III aplasia in control ones. For *in vitro* studies, MDA-231 cells (human breast carcinoma) and HBL-100 (normal mammary epithelium) were irradiated with 0-10 Gy employing the same source. Cell cultures were treated with O-LM for 24 hs previous and up to 24 hs post-irradiation. Number of colonies over 50 cells where counted after 10 days. Cell surviving curves indicated that O-LM produced a significant decrease ($p<0.001$) in survival of transformed cells while no difference was observed in normal HBL-100. These results demonstrated a protective effect on normal tissues exerted by O-LM for *in vivo* high doses of ionizing irradiation and a significant increase in radiosensitivity on the transformed MDA-231 cells but not in normal HBL-100 cells.

## 1. Introduction.

Ionizing radiation, alone or in combination with other therapies, is one of the most used anticancer treatments. It is well know that hematopoyetic toxicity and immune suppression are one of the major problems after irradiation of patients [1]. Injury resulting from the irradiation of biological tissue by ionizing radiation is a consequence of the transfer of radiation energy to critical macromolecules. The initial chemical injury can occur directly by the absorption of radiation or indirectly through the action of free radicals [2]. On the tissue, since water is so abundant, the contribution of free radicals to radiation damage is quite important [2, 3, 4]. Early investigations demonstrate that one of the most important modifiers of radiation damage in biological systems is the oxygen [4]; the oxygen enhances the indirect effect of radiation damage and prevents the repair of damaged molecules by fixing the radiation lesions [2, 3, 4]. More recent experiments indicate that reactive oxygen species (ROS) play an important role in the action of ionizing radiation [5]. Superoxide dismutases (SODs) and gluthatione peroxidase (GPx), are free radical scavenging enzymes that defend cells from oxidant stress. The superoxide ($SO_2$) and the hydrogen peroxide ($H_2O_2$) generated post-irradiation are detoxified by GPxs and SODs. MnSOD (mitochondrial) and CuZnSOD (cytoplasmatic), were reported to be involved in protecting cell against radiation injury [6]. The radioprotective agents appear related to free radicals in competition with oxygen or with increased repair of radiation injury. On the contrary, radiosensitizers are chemical agents that have the capacity to increase the lethal effects of radiation.

The development of drugs that radiosensitize the malignant cell and radioprotect the normal tissues, is yet a challenge for oncologists and radiobiologists. In previous studies we demonstrated the protective effect of the combination of Selenium (Se), Zinc (Zn), Manganese (Mn) plus Lachesis *muta* (O-LM) against carcinogenic drugs and high doses of chemotherapy [7, 8]. This treatment is also applied to

human patients with colon cancer with liver metastases [9], with breast cancer [10], or with pancreatic carcinoma [11], producing in all cases a significant increase in survival rates.

In the present study we evaluated *in vivo* the tolerance induced by O-LM to high doses of ionizing radiation and the *in vitro* effect of O-LM on radiosensitivity of malignant and normal human cell lines.

## 2. Materials and Methods.

### *2.1. Source.*

In all studies, a $^{137}$Cs source of 189 TBq (7.7 Gy/min) calibrated by Argentine National Commission of Atomic Energy with a TLD 700 dosimeter and validated by Argentine Nuclear Regulatory Authority was employed.

### *2.2. O-LM treatment.*

In all studies the medication employed (O-LM) was a combination of the oligoelements Zn, Se, and Mn (4μg/ml each) plus Lachesis *muta* (4ng/ml).

### *2.3. In vivo* studies.

#### *2.3.1. Rats.*

Ninety-six male Sprague-Dawley rats received whole-body irradiation with a single dose ranging from 2 to 15 Gray. The half of animals (48, divided in 8 rats per group) received daily 0.5 ml of O-LM via sc (O-LM group) starting 10 days before irradiation; the treatment continued for 70 days; the other 48 rats were injected daily with placebo. Each group received a single dose of 2, 5, 8, 10, 12 or 15 Gy, respectively. Parameters recorded on these studies were:

- Survival,
- $^{30}$ $DL_{50}$ value,
- Observation of collateral macroscopic effect and body weight determination,
- Histopathology of organs as liver, spleen, kidney, lung, heart.

#### *2.3.2. Nude mice.*

To evaluate the possible protective capability of O-LM, *nude* mice were utilized. The *nude* mice were employed in order to carry further experiments with transplanted human tumours in these mice. Twenty mice were randomly separated in two groups and small intestine and bone marrow were processed post-irradiation to evaluate the histopathological changes.

- Group 1, Control, irradiated receiving sc daily placebo, and
- Group 2, irradiated receiving 0.02 ml sc daily O-LM treatment starting 90 days before irradiation.

All mice received a single whole-body dose of 10 Gy and were sacrificed 5 days after irradiation. Parameters analyzed in these studies were:

- Number of intestinal crypt,
- Villous edema,
- Ulceration,
- Vascular damage,
- Bone marrow aplasia.

The small-intestine specimens were pinned flat on corkboard and immediately fixed. Haematoxylin-Eosin (H.E.) stained sections were examined for mucosae highness and crypt number per circumference evaluation. Histological and cytological condition of intestinal villi was carefully

evaluated. Nuclear changes (anisocariosis, macronuclei, picnosis), anisocytosis, cell ballooning, interstitial edema, mucosae exulceration and vascular damage signs were registered and compared in both groups.

### *2.4. In vitro studies.*

*2.4.1. Cell survival fraction.*

MDA-231 cells (human breast carcinoma) and HBL-100 (normal mammary epithelium) were cultured in RPMI 1640 medium (Gibco BRL, Grand Island Biological Co, NY) supplemented with 10% of calf serum (Gibco BRL), glutamine 0,3 g/l (Sigma Co, Saint Louis, USA) and gentamicine 0,04 mg/l. To carry on radiosensitivity studies 3300 cells were seeded in plastic flasks and after 24 hrs were irradiated with doses ranging from 0 to 10 Gy. Eight days post irradiation the clonogenic proliferation was evaluated by counting the colonies containing 50 cells or more. Colonies were fixed with 10 % buffered saline-formaline solution and stained with Toluidine Blue (T.B.). For cell treatment, 0.02 ml of O-LM per ml of culture medium was added to flasks immediately after plating and continued up to 24 hrs post irradiation. The parameters analyzed were:

- Survival curve, and
- Radiosensivity parameters (alpha, beta, alpha/beta, survival fraction at 2 Gy and Doses that reduce survival fraction at 0.01.

Survival curves were graphed and adjusted to the Linear-quadratic mathematical model [3] employing the GraphPrism program.

## 3. Results and Discussion.

### *3.1. In vivo results.*

*3.1.1. Rats.*

*3.1.1.1.Survival.*

The results obtained indicate that O-LM animals receiving 8, 10, and 12 Gy (figure 1a, 1b and 1c, respectively) presented significantly higher survival versus controls ($p<0.0001$, 0.003 and 0.0083, respectively, longrank test). Global survival of O-LM treated rats, also showed a significantly higher value vs Control ($p<0.0005$, logrank test) (figure1d).

3.1.1.2. Determination of $^{30}DL_{50}$ value.

$^{30}LD_{50}$ value for O-LM treated rats was 9.6 Gy vs. 5.8 Gy for control ($p<0.0001$, two way ANOVA). These values clearly indicate a protective action of O-LM in treated animals (figure 2).

*3.1.1.3. Collateral effects and Body weight determination.*

No collateral effect was seen in rats treated with O-LM and irradiated with 2 or 5 Gy. The figure 3 clearly illustrate that rats receiving O-LM and irradiated with 2 Gy show a recovery of body weight significantly higher than control ($p<0.0001$, two way ANOVA test).

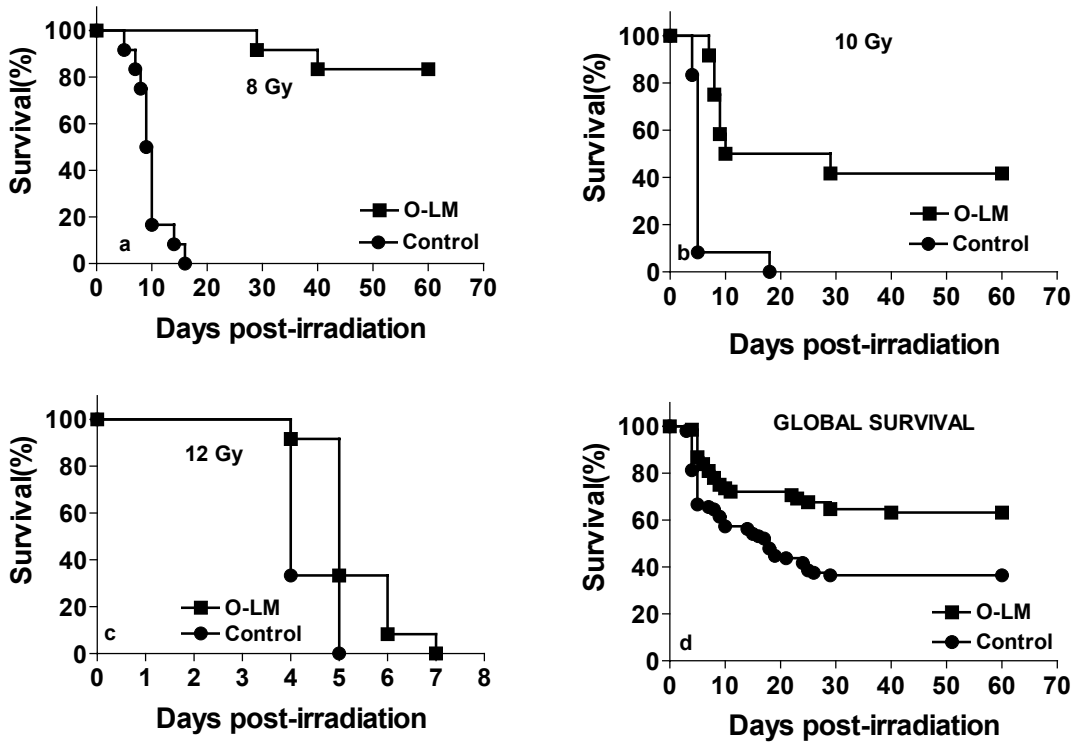


***FIG 1***. *Survival of rats (O-LM treated vs Control) whole body irradiated with different Dose.*

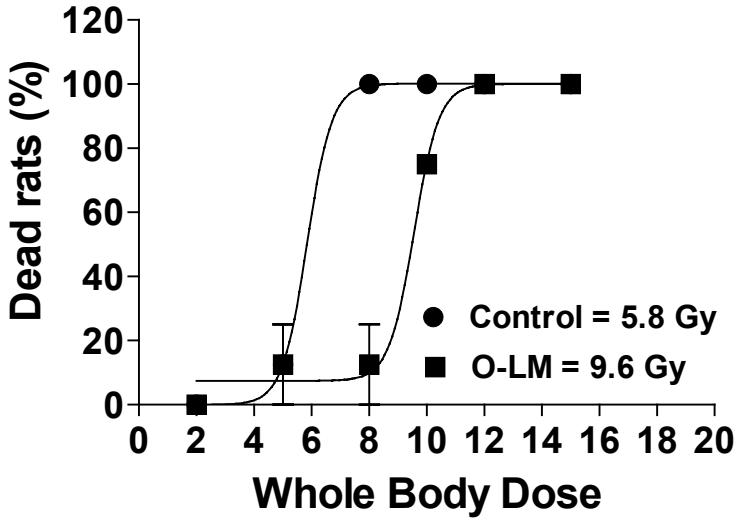


***FIG 2.*** *$^{30}LD_{50}$ value of O-LM vs. Control rats.*

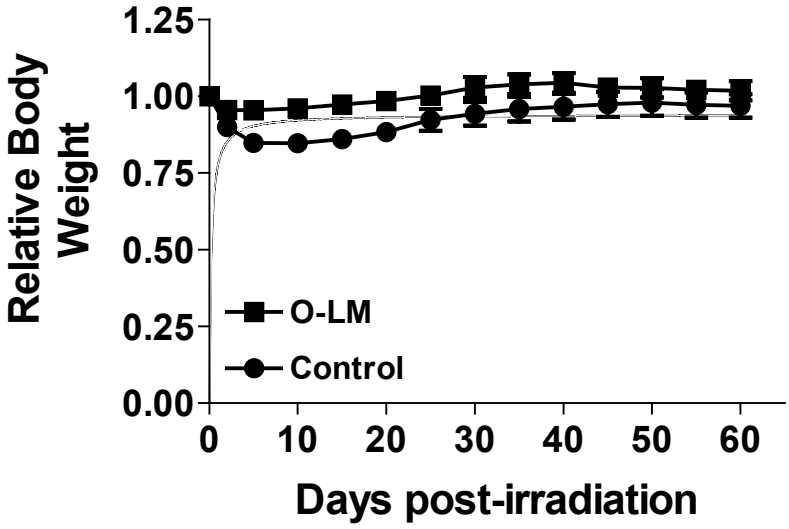


***FIG 3.*** *Relative Body weight of rats O-LM treated vs. Control, irradiated with 2 Gy.*

*3.1.1.4. Histopathology of organs.*

Anatomopathological alterations involving bone marrow, kidney and liver were found. Histopathological studies indicated that 100% of the rats presented severe spleen atrophy specially of lymphoid related structures but also the sinusoidal sectors were affected. All the animals presented severe bone marrow aplasia usually grade III in the control groups, instead the O-LM treated animal showed mainly Grade II or Grade I-II bone marrow aplasia. Acute renal tubular necrosis was seen in some cases and cellular hepatic lesions were also evident.

*3.2. Nude mice.*

Organic manifestations of the radiation damage were scarce; only an important loss of lymphoid tissue of the spleen, ballooning and macronuclei in hepatocytes and slight irritative tubular renal lesions were seen. Some degree of vascular damage may surely be present but the short time between irradiation and the termination of the experiment may be responsible of the scarce histological findings in the studied organs.

The intestinal mucosae of mice of group 1, (irradiated with 10 Gy) showed severe degenerative changes characterized by decreased villous height, reduction of crypt number, villous edema, ulceration, vascular damage, anisocariosis and cytoplasmic alterations. In contrast, the mucosae of animals of group 2, irradiated with 10 Gy and receiving daily O-LM, showed higher number of crypts, better villous conservation, lack of edema and of vascular damage and showed less nuclear and cytoplasmic alterations (Table I).

**Table 1. Comparative histopathological changes observed in control and O-LM treated mice.**

| Autopsy results | Small bowel mucosal trophism | Average N° of crypts/ 0,5 cm | Mucosal exulceration | Nuclear and cytoplasmic changes | Villous edema | Vascular damage |
|---|---|---|---|---|---|---|
| Control | Marked atrophy | 75 | Severe | Anisocariosis, anisocitosis | Present | Present |
| O-LM | Moderate atrophy | 137 | Mild | Mild anisocariosis (conservation of Panneth cells) | Absent | Absent |

The markedly alteration on intestinal mucosae of nude mice irradiated with 10 Gy can be seen. On the contrary, mice receiving O-LM treatment show conservation of the normal number of crypts and intestinal villi (figure 4).

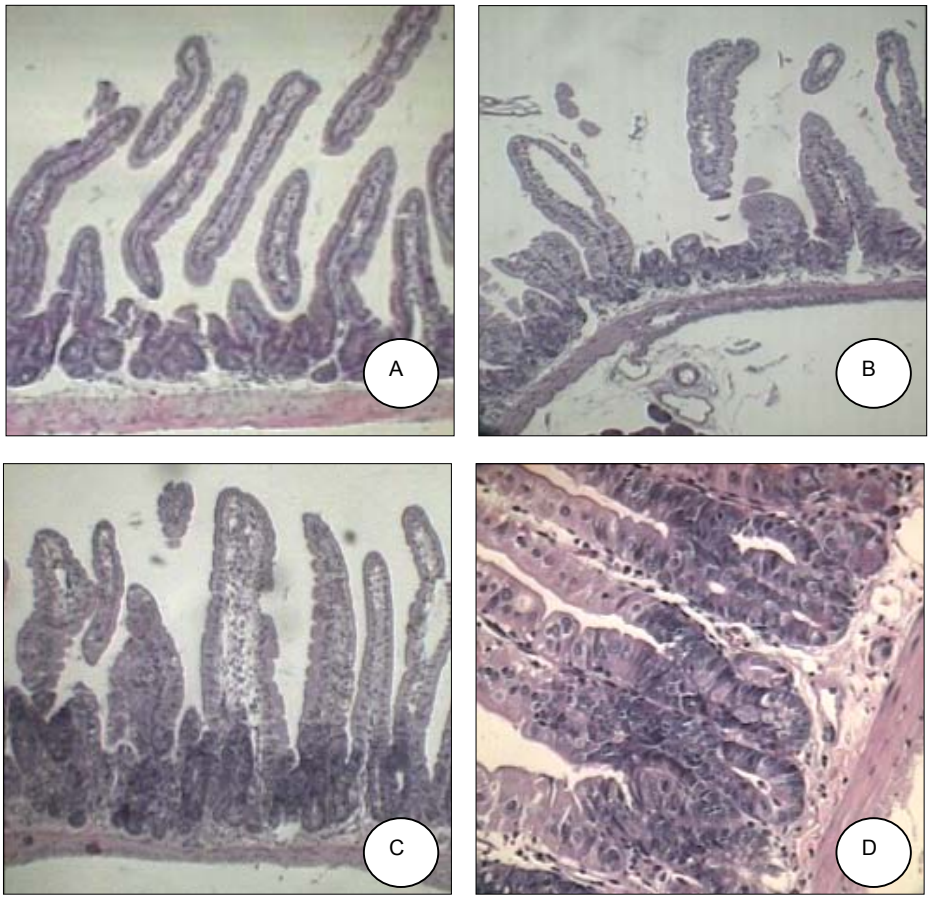


***FIG 4.*** *A) Normal looking intestinal mucosae of control non irradiated nude mice (H.E. 20X). B) Markedly altered intestinal mucosae of 10 Gy irradiated nude mice, reduced number of crypts, disorganization of the remaining villi, edema and loss of entire sectors of columnar epithelium can be seen (H.E. 20X). C) 10 Gy irradiated O-LM pretreated nude mice showing conservation of crypts and villi. Only slight edema and scarce epithelium loss can be seen. (H.E. 20X). D) 10 Gy irradiated O-LM pretreated mice showing some grade of anisocitosis and anisocariosis of the mucosal cells (HE 100X).*

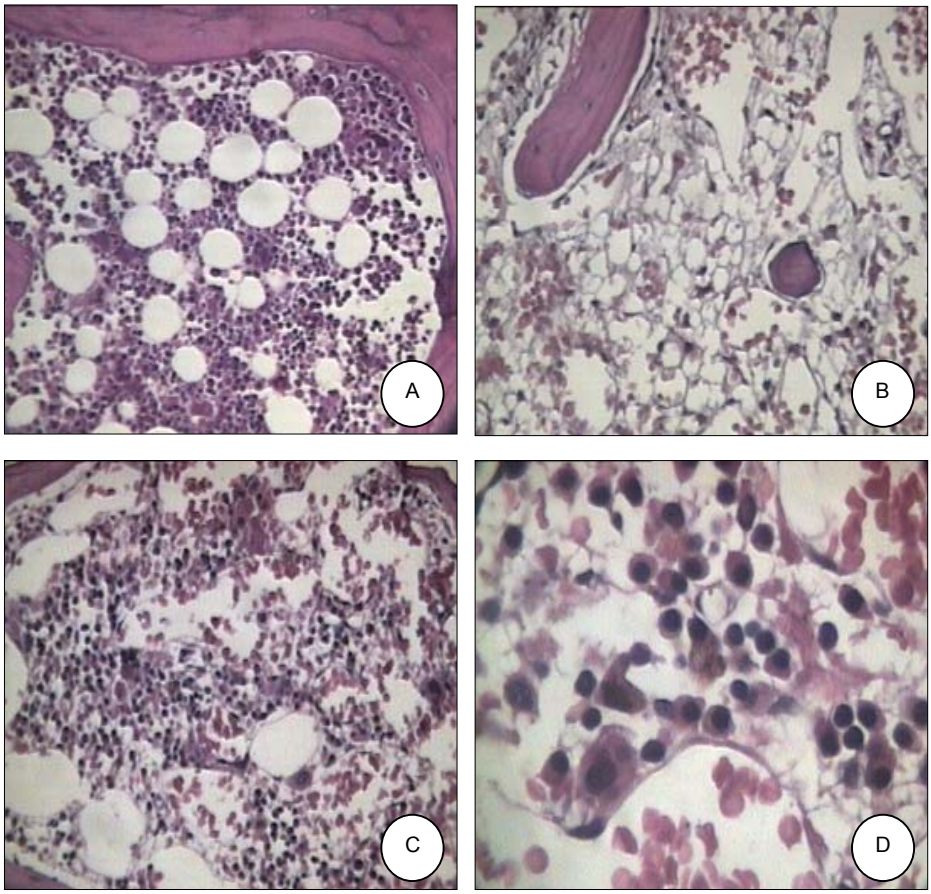


***FIG 5.*** *A) Normal bone marrow of a non-irradiated nude mice (H.E. 100X). B) Grade III (total) medullar aplasia in a 10 Gy irradiated nude mice (H.E. 100X). C) Bone marrow of a 10 Gy irradiated O-LM pretreated nude mice showing partial conservation of medullar cell lines and also partial hemorrhagic replacement of the lost elements; grade I-II aplasia (H.E. 100X). D) Bone marrow of a 10 Gy irradiated O-LM pretreated nude mice with partial conservation of granulocitic, megacariocitic and eritroblastic cell lines (H.E. 400X).*

Also, important differences were observed on bone marrow of O-LM mice versus Control ones (figure 5).

### *3.2. In vitro results*

#### *3.2.1. Cell survival fraction*

Survival curves obtained with the human cell lines indicate that MDA-MB-231 breast carcinoma cells are moderately radiosensitive according to the calculated parameters (Table II). The treatment with O-LM significantly increased cell radiosensitivity decreasing the survival fraction at 2 Gy from 0.25±0.07 in controls to 0.14±0.03 in treated cells ($p<0.001$ ANOVA). On the contrary in the HBL-100 normal cells the treatment with O-LM did not modified the calculated parameters and the survival fraction, in coincidence with the results obtained *in vivo* that showed a protective effect of O-LM in normal tissue (figure 6).

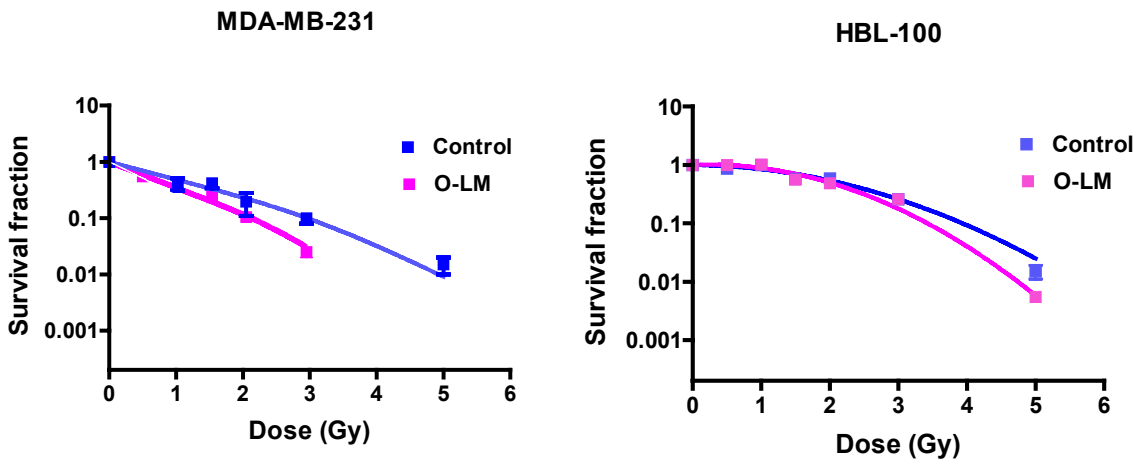


***FIG 6.*** *Survival curves obtained with the human cell lines MDA-MB-231 and HBL-100, treated and non-treated with O-LM.*

*3.2.2. Radiobiological parameters*

**Table II. Radiobiological Parameters**

| Cell line | α (Gy $^{-1}$) | β (Gy $^{-2}$) | α/β (Gy) | SF $_{2Gy}$ | SF $_{2Gy}$ (O-LM) | Dose $_{0,01}$ (Gy) |
|---|---|---|---|---|---|---|
| MDA-MB-231 | 0.53±0.08 | 0.60±0.02 | 8.83 | 0.25±0.07 | 0.14±0.03 [a] | 5.2±0.8 |
| HBL-100 | 0.05±0.01 | 0.16±0.04 | 0.31 | 0.54±0.11 | 0.53±0.10 | 5.0±0.1 |

*Table II. MDA-231 and HBL-100 cells irradiated with 10 Gy.* [a] *$p<0.01$ irradiated and treated with O-LM vs. irradiated control.*

**4. Conclusions.**

The present studies demonstrated a protective effect on normal tissues exerted by O-LM for *in vivo* high doses of ionizing radiation. In addition, O-LM produced a significant increase in radiosensitivity only on the transformed MDA-MB-231 cells but not on normal HBL-100 cells. Experiments carried out in our laboratory clearly show that the treatment with O-LM modulates MnSOD activity as well as superoxide and hydrogen peroxide intracellular levels. This effect may represent the basis of the mechanism responsible for the selective protection against ionizing radiation.

The ability of radioprotectores to produce their effect is a result of their capacity to inhibit indirect damage, to repair direct or indirect effects when occur, and to facilitate the recovery of damaged cells or depleted cell population. In that sense it was evident the protective effect of O-LM treatment on

normal medullar cell in both rats and mice. In histopathological studies a lower grade of medullar aplasia was observed in O-LM treated animals; in the intestinal mucosae of treated mice the loss of crypts and normal intestinal villi was markedly lower than in control animals. These findings prove the protective effect on two highly radiosensitive normal tissues.

Based on these results we propose that O-LM may be considered a potentially clinical useful radioprotectant with the possibility of applying this protective treatment to human patients undergoing radiotherapy.

**5. References.**


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