

# Deleterious effect on immunity produced by ionizing radiation; protective effect of a novel therapeutic formulation composed by Zn, Se and Mn plus Lachesis Muta venom (O-LM)

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**Abstract.** High doses of ionizing radiation exert deleterious effects on immunity. Previously we demonstrated that O-LM, a novel therapeutic combination of three oligoelements (Zinc, Selenium, Manganese) and Lachesis muta venom, inhibits malignant cell proliferation and increases survival in rodent tumor models. O-LM effects involve both anti-oxidant action and up-regulation of innate and T-cell mediated immunity. Moreover, O-LM selectively protected normal tissues as small intestine or bone marrow, from high doses of ionizing radiation or chemotherapeutic drugs. We here investigated the protective action of O-LM on radiation effects upon immune cells. BALB/c mice were divided in 3 groups (n=15): control (C), 2Gy whole body irradiated (IRRAD) and 15-days O-LM treated and irradiated (O-LM+IRRAD). Mitogen-induced T and B lymphocyte proliferation, as well as key cytokine involved in lymphocyte regulation and/or inflammation were evaluated at different days post-irradiation (PI). We found that irradiation induced a decrease in T lymphocyte proliferation at 3 and 7 days PI (% of decrease: 47.6±9.0, p<0.05; 42.0±7.2, p<0.02 respectively). Pretreatment with O-LM recovered proliferation to basal values (day 3 PI 93.4±10.2%; day 7 PI 130.9±15.3%). No modifications were observed in B cells. Also at day 3 PI, a marked decrease in IFN $\gamma$  levels was obtained in supernatants from mitogen-stimulated lymphocyte cultures from IRRAD mice; this effect was reverted by O-LM treatment (pg/ml: IRRAD: 1653±419; C 10884±2783, p<0.02; O-LM+IRRAD 16924±4284, p<0.05 vs C). At day 7 PI, an important increase in TNF $\alpha$  was observed in IRRAD mice, that was reverted by O-LM (pg/ml: IRRAD: 300.7±62.3 vs O-LM+IRRAD: 28.7±2.3, p<0.02). No differences were found in IL-2 levels. We conclude that O-LM protects animals from irradiation by recovering the immune function, improving T lymphocyte activity and modulating the production of key cytokines as IFN $\gamma$  and TNF $\alpha$ . The possibility that these effects would be related to O-LM anti-oxidant properties is now under study. The reported effect may represent a potential benefit for cancer patients undergoing radiotherapy.

**KEYWORDS:** *Ionizing radiation, Irradiation, Immunity, Lymphocyte, Cytokine*

## 1. Introduction

Ionizing radiation (IR) therapy is an important local modality for the treatment of cancer. The current rationale for its use is based largely on the ability of IR to kill the cancer cells by a direct cytotoxic effect. This biologic action is mainly exerted through the induction of double strand breaks to DNA in order to induce elimination of cancerous cells, but indirect mechanisms related to free radical production are also involved. Additionally, IR induces multiple responses in target cells as a consequence of the activation of numerous signaling cascades and through the release of clastogenic factors including lipid peroxidation products, cytokines and other oxidants with chromosome-damaging properties [1-3]. Considerable evidence indicates that IR effects extend beyond the mere elimination of the more radiosensitive fraction of cancer cells present within a tumor at the time of radiation exposure. The ability of IR to modify the tumor microenvironment and generate inflammation was demonstrated [4, 5]. Administration of radiation has been utilized to create an inflammatory setting, via induction of apoptosis, necrosis, cell surface molecules, and secretor molecules. Caspase-mediated cellular apoptosis is induced by radiation through multiple signaling pathways [6].

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Ionizing radiation has a well-established ability to kill cancer cells and other cells within the tumor stroma, including endothelial cells and intratumoral lymphocytes [3]. Tumor cells killed by IR should be a very good source of antigens for dendritic cells uptake and presentation to T cells [7]. The possibility that antitumor immunity can be elicited *in vivo* when tumor irradiation was suggested, but, no direct evidence that IR on its own is able to enhance tumor immunity is currently available. Optimal activation of T cells by dendritic cells presenting tumor-derived antigens can be achieved only in the presence of inflammatory signals [1, 8-10].

Conventional cytotoxic therapies, such as radiation and chemotherapy, have been generally viewed as immunosuppressive. However, advances in the understanding of the mechanisms that regulate the development of antitumor immunity, as well as improved knowledge of the complex effects of radiation on tissues [1], have revived interest in the possibility of combining radiation and immune-based therapies to achieve better local and systemic tumor control [6].

By the other hand, a novel therapeutic combination of oligoelements (O, namely, Zinc, Selenium and Manganese) and *Lachesis muta* (LM) venom, O-LM, was demonstrated to inhibit malignant cell proliferation and to increase survival in rodent tumor models [11, 12]. We have recently shown that this formulation is also able to stimulate the immune response in immunocompetent mice [13, 14]. These actions were mediated in part due to its anti-oxidant action and additionally by its capacity of producing an up-regulation of innate and T-cell mediated immunity. Also we have reported that O-LM selectively protects normal tissues as small intestine or bone marrow, from high doses of ionizing radiation or chemotherapeutic drugs [11].

Taking into account the above mentioned evidences, the purpose of the present study was to investigate the potential protective action of O-LM on radiation effects upon immune cells.

## **2. Materials and methods**

### **2.1 O-LM Composition**

As previously described the novel therapeutic formulation under study is composed by Zn, Se and Mn 4 µg/ml of each and *Lachesis muta* 4 ng/ml, in saline.

### **2.2 Irradiation Source**

For animal irradiation (whole body single dose) a source of <sup>137</sup>Cs of 189 TBq and dose rate 7.7 Gy/min (CEBIRSA, Buenos Aires, Argentina) was used. This equipment (IBL 437C H type, number 90330) is periodically calibrated and certified by the Nuclear Regulatory Authority of Argentina, with a TLD 700 contained in polyethylene capsules.

### **2.3 Animals and treatments**

Female mice from inbred BALB/c strain (Instituto Nacional de Tecnología Agropecuaria, INTA, Argentina) were used throughout. At the beginning of the experiments animals were 60 days old. Animals were housed in standard conditions of light and temperature and their care was in accordance with the with the principles and guidelines published in the Guide for the care and use of Laboratory Animals, US National Research Council, 1996. Animals were divided in 3 groups (n=15 each) and received the following treatments: a) vehicle (controls, C); b) exposure to 2Gy irradiation (IRRAD); c) treated first with O-LM, 0.1 ml/day, sc for 15 days and the irradiated with 2 Gy.

### **2.4 Preparation of lymphocyte suspensions and culture conditions**

Aseptically prepared lymphoid cell suspensions were obtained of lymph nodes from animals of different experimental groups female 60-90 days old BALB/c inbred mice (Instituto Nacional de Tecnología Agropecuaria (INTA) as described before [15]. Cells, at a concentration of 1 x 10<sup>6</sup>

cells/ml, were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL), 2 mM glutamine (GIBCO BRL) and antibiotics (GIBCO BRL). Cells were settled at a final volume of 0.2 ml in 96-well flat-bottom microtiter plates (Nunc TM) and were cultured for 72 h, alone (Basal) or in the presence of increasing concentrations of T selective, concanavalin A (Con A), or B selective, Pokeweed Mitogen (PWM) or lipopolysaccharide (LPS), as previously described [15, 16]. All these mitogens were from SIGMA Chemical Co.

## 2.5 Proliferation assays

Proliferation was evaluated on cell cultures by pulsing cells with [<sup>3</sup>H]-thymidine ([<sup>3</sup>H]TdR, NEN, 20 Ci/mmol) for the last 12 hs of incubation, as described before [15, 16]. Results are expressed as dpm values in experimental cultures subtracting the dpm control values. Cultures of unstimulated cells were used as controls.

## 2.6 Determination of cytokine levels:

Levels of IFN- $\gamma$ , IL-2, IL-6 and TNF- $\alpha$  were determined with commercial ELISA kits (Pierce) in supernatants from lymph node or spleen cell cultures from mice of the three experimental groups described above and stimulated for 24 hours with Con A (1  $\mu$ g/ml).

## 2.7 Statistic Analysis:

Results were analyzed with Student t'test or ANOVA followed by the Dunnet's test for determining significant differences.

## 3. Results and Discussion

### 3.1 Mitogen-induced lymphocyte proliferation in irradiated mice treated or not with O-LM at different days post-irradiation:

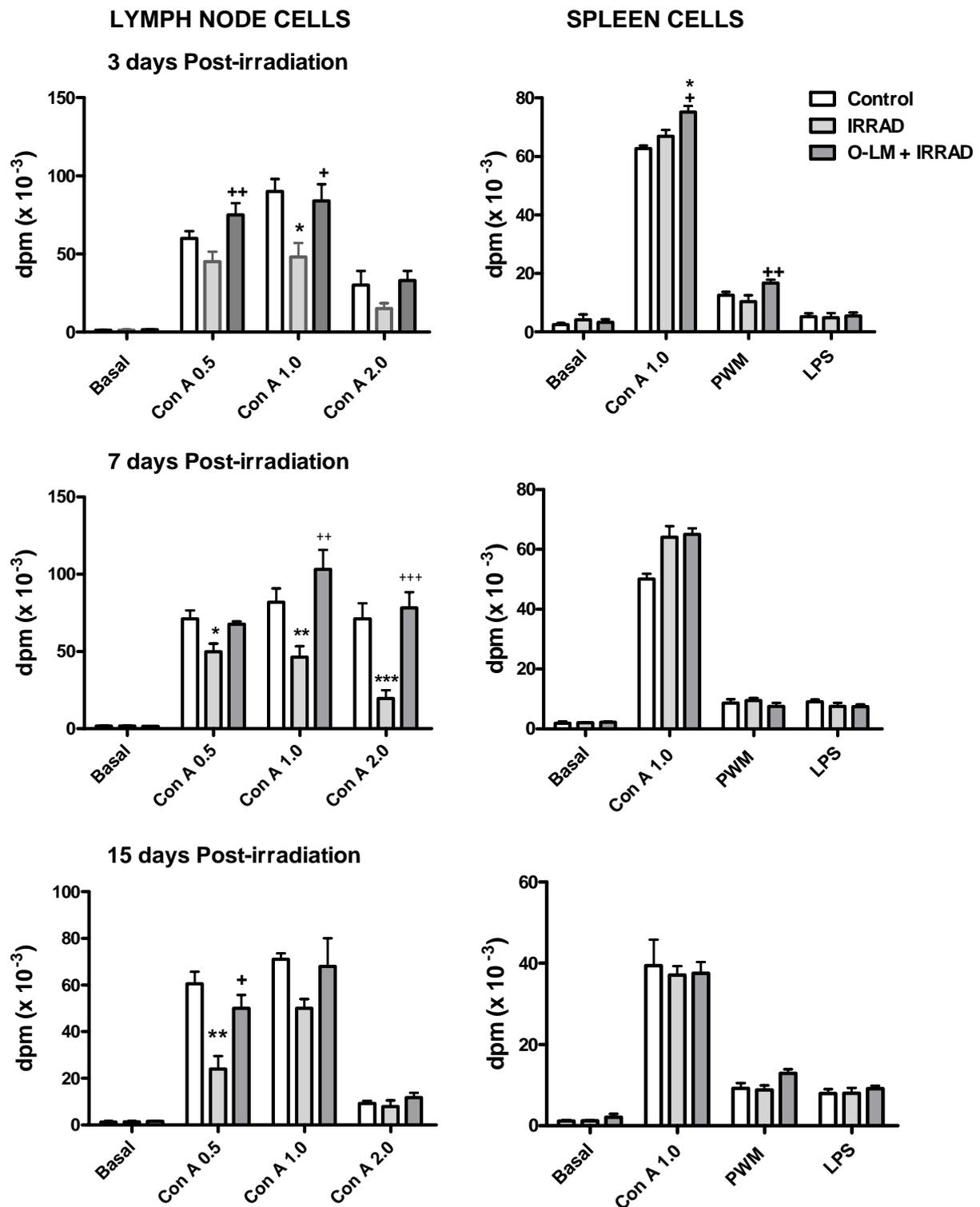
To evaluate O-LM protective action on IR effect on lymphocyte reactivity, mitogen-induced proliferation of lymphocytes from treated or untreated O-LM irradiated mice were evaluated. For this purpose, mice treated or not with O-LM for 15 days, were irradiated as indicated before. At different days post-irradiation (3, 7 and 15 days) lymph node or spleen lymphocytes were obtained and stimulated in vitro with the T and/or B selective mitogens indicated in the figures. Results shown in Figure 1 (left panels) for lymph node cells stimulated with increasing concentrations of Con A, indicate that irradiation induced a decrease in T lymphocyte proliferation at 3 and 7 days PI (% of decrease in IRRAD: 47.6 $\pm$ 9.0, p<0.05; 42.0 $\pm$ 7.2, p<0.02 respectively for Con A 1 $\mu$ g/ml). This effect is observed a few days after irradiation and animals seem to recover after 15 days. Pretreatment with O-LM recovered proliferation to basal values (day 3 PI 93.4 $\pm$ 10.2%; day 7 PI 130.9 $\pm$ 15.3%, O-LM+IR vs. C; p=NS). Right panels in Figure 1 show the results of spleen cell stimulation with T or B selective mitogens. No modifications were observed in B cells with irradiation.

### Figure 1: Effect of irradiation and O-LM pre-treatment in T or B mitogen-induced proliferation of lymph nodes and spleen cells at different days post-irradiation

Left panels show results for lymph nodes cells obtained from animals of the three experimental groups after different days post-irradiation and stimulated with increasing concentrations of Con A. Results shown are the mean  $\pm$  SEM of n = 5 animal in each group. Differs from control with: \* p<0.05; \*\* p<0.02; \*\*\* p<0.001; differs from irradiated (IRRAD) with: + p<0.05; ++ p<0.02; +++ p<0.001.

Right panels show results for spleen cells obtained as above and stimulated with T or B-selective mitogens, namely, Con A (1  $\mu$ g/ml), PWM (5  $\mu$ g/ml) or LPS (30  $\mu$ g/ml). Results shown for different

days post-irradiation are the mean  $\pm$  SEM of  $n=5$  animal in each group. \*Differs from control with  $p<0.05$ ; differs from irradiated (IRRAD) with: +  $p<0.05$ ; ++  $p<0.01$



### 3.1 Cytokine released upon mitogen stimulation in cell free supernatants of lymphocytes from treated or untreated O-LM irradiated mice at different days post-irradiation:

To ascertain irradiation alteration of T lymphocyte reactivity and O-LM reversal effect were accompanied by the regulation of the level of cytokine-mediators, key cytokines involved in T-cell regulation and/or inflammation were evaluated. The following cytokines, IFN- $\gamma$ , IL-2, IL-6 and TNF- $\alpha$

were determined by ELISA in cell-free supernatants from cultures of mitogen-stimulated lymphocytes from the experimental groups described before. At day 3 post-irradiation, a marked decrease in both IFN $\gamma$  and IL-6 levels were obtained in supernatants of irradiated mice, which were reverted by O-LM treatment. Also, at day 7 PI, an important increase in TNF $\alpha$  was observed in irradiated mice respect to control that was also reverted by O-LM. These effects were found a few days after exposure to IR and were then compensated in vivo as at 15 days post irradiation no changes were observed both for cell proliferation and for stimulated cytokine production. It is worth noting that no differences were found in IL-2 levels at any time studied.

**Table 1. Cytokine levels after different days post-irradiation in cell free supernatants from lymph node cells stimulated with Con A:**

Days PI	Treatment (a)	IFN- $\gamma$ (pg/ml)	IL-2 (pg/ml)	IL-6 (pg/ml)
3	Control	10884 $\pm$ 2783	8306 $\pm$ 1881	1205 $\pm$ 95
	IRRAD	1653 $\pm$ 419*	8093 $\pm$ 719	535 $\pm$ 82*
	O-LM + IRRAD	16924 $\pm$ 4284 <sup>+</sup>	11381 $\pm$ 1783	1687 $\pm$ 73 <sup>++</sup>
7	Control	25732 $\pm$ 4101	5917 $\pm$ 2609	1206 $\pm$ 86
	IRRAD	24058 $\pm$ 2771	10730 $\pm$ 4037	728 $\pm$ 30
	O-LM + IRRAD	23353 $\pm$ 1728	12311 $\pm$ 6066	985 $\pm$ 100

(a) Cytokines were measured in supernatants from  $5 \times 10^6$  cells obtained from lymph nodes and stimulated with Con A (1  $\mu$ g/ml). Results shown are the mean  $\pm$  SEM of n=5 animals of each group, determined by duplicate. \* Differs from control with p<0.05; + differs from IRRAD with p<0.02.

**Table 2. Cytokine levels after different days post-irradiation in cell free supernatnas from spleen cells stimulated with Con A:**

Days PI	Treatment (a)	IFN- $\gamma$ (pg/ml)	IL-2 (pg/ml)	TNF $\alpha$ (pg/ml)	IL-6 (pg/ml)
3	Control	47560 $\pm$ 14400	8259 $\pm$ 2670	12.5 $\pm$ 5.0	2152 $\pm$ 50
	IRRAD	53280 $\pm$ 9672	6956 $\pm$ 1254	20.5 $\pm$ 8.5	1240 $\pm$ 68 *
	O-LM + IRRAD	52226 $\pm$ 5447	7262 $\pm$ 270	< 12.5	1758 $\pm$ 155
7	Control	81940 $\pm$ 11930	18874 $\pm$ 6225	< 12.5	2588 $\pm$ 20
	IRRAD	106465 $\pm$ 16635	24388 $\pm$ 6604	300.7 $\pm$ 62.3*	2135 $\pm$ 106
	O-LM + IRRAD	155950 $\pm$ 15457 * <sup>+</sup>	39912 $\pm$ 6312	28.7 $\pm$ 2.3 <sup>++</sup>	1867 $\pm$ 64
15	IRRAD	88449 $\pm$ 7431	11617 $\pm$ 883	163.8 $\pm$ 90.2	2254 $\pm$ 25
	O-LM + IRRAD	138443 $\pm$ 9290 <sup>++</sup>	11055 $\pm$ 675	66.4 $\pm$ 15.2	2272 $\pm$ 128

(a) Cytokines were determined in supernatants of  $5 \times 10^6$  cells from spleen, stimulated with Con A (2  $\mu$ g/ml). Results shown are the mean  $\pm$  SEM of n=5 animals of each group, determined by duplicate. \* Differs from the corresponding control with p<0.02; + differs from IRRAD with p<0.05; ++ differs from IRRAD with p<0.02.

Results for irradiated animals are in agreement with previous findings indicating attrition of T-cell functions, reductions in T mitogen-dependent proliferation, decrease in helper T-cell populations and

increase in blood inflammatory cytokine levels after whole body exposure to IR [17, 18]. Moreover, it was previously demonstrated that radiation is not only able to induce the proinflammatory cytokines IL-1 $\beta$  and TNF $\alpha$ , both in vitro and in vivo [10, 19], but also to reduce the Th1 cytokine IFN $\gamma$  as well, thus suggesting that IR reduces the Th1 like function, resulting in a Th1/Th2 imbalance [20, 21].

Treatment of animals with O-LM reverted irradiation effects upon T cell proliferation and cytokine production and this would be related to the stimulating effects of this formulation on the immune responses we have previously described [13, 14]. Our results indicate that O-LM protects against radiation-induced damage by stimulating the proliferation and maturation of immune cells. This stimulatory effect of O-LM on T cells may promote the synthesis of cytokines. Similarly Zinc and other immunostimulant natural products were also able to lead to the improvement of irradiation-induced immunosuppression [22-24].

Finally, radiation-induced destruction of the lymphoid systems causes the loss of the immune function. Subsequently, the exposed individuals become susceptible to opportunistic pathogens or to secondary tumors. Therefore, the immunostimulatory potential of O-LM is an important component of its radioprotective efficacy. It is important to note that O-LM reverted the irradiation induced inhibition on IFN- $\gamma$  and IL-6. The first is a key cytokine involved in T-cell mediated anti-tumoral immunity [25]. On the other hand it has been documented the important rol for IL-6 in both the resolution of innate immunity and the development of acquired immune responses [26]. Also, the O-LM effect decreasing TNF $\alpha$  levels together with IL-6 increase could represent a mechanism to reduce inflammatory processes induced by irradiation. However, the possibility that these effects would also be related to O-LM anti-oxidant properties can not be ruled out and are now under study. Nevertheless the exact mechanism involved, we propose that O-LM is a candidate for adjuvant therapy to alleviate radiation-induced damage to cancer patients.

#### **4. Conclusions**

The present data demonstrate that O-LM protects animals from irradiation by recovering the immune function, improving T lymphocyte activity and modulating the production of key cytokines as IFN $\gamma$  and IL-6. These effects would be related to O-LM immunostimulant actions. Moreover, O-LM could also regulate the inflammatory response to irradiation. Finally, the possible involvement of O-LM anti-oxidant properties can not be discarded. The reported effect may represent a potential benefit for cancer patients undergoing radiotherapy.

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