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Protection of Mice against X-ray Injuries by the Post-irradiation Administration of Inosine-5'-monophosphate

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Inosine-5'-monophosphate/Ionizing radiation/Mitigator/Radioprotector.

The aim of the present study was to investigate the radiation modulating properties of inosine-5'-monophosphate (IMP). Mice injected introperitoneally (i.p.) with IMP 15 minutes after irradiation with a lethal irradiation dose of 7 Gy have better survival rates comparative to irradiated mice non treated with IMP. The dose reduction factor of the IMP is 1.22. Using a hematologdical test we demonstrated that administration of IMP alleviates the symptoms of radiation-induced leukopenia and thrombocytopenia. The DNA damage in bone marrow and thymus cells of irradiated mice was measured by flow cytofluorometry and micronucleus test (MN-test). The tests show that i.p. administration of IMP to irradiated animals leads to a significant reduction of the DNA damage level. In this paper we show that IMP substantially modulates the damaging effects of ionizing radiation protecting irradiated mice and it is a promising agent for a treatment of leukopenia.

INTRODUCTION

First chemical compounds that diminish damaging effects of ionizing radiation on laboratory animals were discovered more than half a century ago.[1)] This protective effect was termed as "chemical protection against radiation injury". The very chemical compounds, exerting protective effect, received a name "radioprotectors". As a rule, the radioprotective effect exists, when the radioprotectors are introduced into the body shortly before irradiation (usually 10–30 minutes).[2)] Also, the term "mitigator" is often in use. Mitigators are given during radiation exposure or shortly after exposure, before the appearance of overt evidence of an injury.[3)] There are two known major classes of mitigators: purine nucleoside analogs (RNA extracts, hydrolyzates of RNA, guanosine, GMP and inosine) and growth factors (G-CSF, IL-2, CSF).[4)] It was established, that protective properties of purines increase in the order: guanosine ≤ GMP ≤ inosine.[5,6)] We supposed, that the protective potential of IMP will be more pronounced than that of the inosine. At the present time radioprotective and mitigate properties of IMP have not been studied in detail. To the best of our knowledge there is only one research showing that IMP administration has been protective, resulting in lowered mean skin reaction observed on the local irradiated hind legs of rats.[7)] Weissber and Fischer[7)] have shown that skin protective properties of IMP were more pronounced than that of the inosine. This effect is an indirect confirmation of our assumption that IMP is a most effective radioprotective compound in comparison with other previously studied purines. In connection with our suggestion, the purpose of this study was to investigate the mitigate properties of IMP.

MATERIALS AND METHOD

Irradiation

Animals were whole-body irradiated on a RUT-15 therapeutic X-ray unit (Mosrentgen, Moscow, Russia) with doses of 7 Gy or 1,5 Gy (1 Gy/min, focal distance 37.5 cm, current 15 mA, voltage 200 kV) at a room temperature.

Animals

Male white outbred mice (Kv:SHK) were used in all experiments. The animals were keep in vivarium in polypropylene cages with sawdust as a bedding material and maintained under temperature controlled conditions (22 ± 3°C) with duration of daylight (12/12 hours). Mice were given standard commercial mouse feed (Arno, Russia) and drinking water *ad labium*. For all experiments five-week-old male

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mice weighing 17–20 g were used. Animal handling was done according to institutional guidelines for animal care. All the experimental protocols received approval from the Bioethics Committee of the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences.

Survival of mice

IMP was dissolved in 0.14 M NaCl to a concentration of 5 mM right prior to an experiment. Mice were administrated with intraperitoneal (i.p.) IMP solution 15 min after irradiation with a dose 7 Gy. Animals were observed for 30 days after the irradiation, and the number of surviving mice was checked at the same time every day. Body weight changes and the amount of food and water consumed by the animals were measured once in two-three days. The technique was described in more details previously.[8)]

Blood cell count

Groups of 10 mice were originally used. Five mice from each group were randomly taken for blood cell count, and then the results were averaged for these five animals. At the end of the experiment, if the number of survived mice in each group was less than five, all the mice were taken for blood draws. Samples of peripheral blood were taken from a tail vein of mice. All experimental procedures were described in details previously.[9)]

Micronucleus (MN) test

The IMP solutions (45 mg/kg in 0.14 M NaCl, 0,5 ml/animal) was i.p. administrated to mice 15 min after the irradiation with a dose of 1.5 Gy. This dose value was chosen as the most optimal, since the "dose-effect" linear dependence for mice Kv:SHK was manifested in a dose range 0–2 Gy.[10)] Mice were destroyed by cervical dislocation 24 h after irradiation because a maximum yield of polychromatic erythrocytes (PCE) with micronuclei (MN) was detected approximately one day after exposure to ionizing radiation. Procedures related to the preparation and staining of histological samples were described previously.[9)] Counting the PCE-containing MN was performed using a light microscope MikMed-2 (LOMO, Russia) with an immersion lens with a magnification × 1000. The data for 5 animals per one experimental point were analyzed, with minimal count cells 2000 per mouse sample.

Flow Cytofluorometry

Mice were administrated i.p. IMP (45 mg/kg in 0.14 M NaCl 0,5 ml) 15 min after irradiation (7 Gy). Thymus cells extraction was performed as described in Ormerod[11)] in 22 hours after irradiation.[6)] Cells were measured on Partec PAS III cytofluorimeter (Germany).

Statistical analysis

Mean and standard error of the mean (SEM) were calculated for most variables. The means from the different treatment groups were compared by the Mann-Whitney U test or Student's unpaired t-test when appropriate. In the survival experiments, the survival curves of different groups were compared by Fisher's exact test. Statistical significance was assigned to $p < 0.05$.

RESULTS

Intraperitoneal post-irradiation administration of IMP in concentrations 15, 45 and 90 mg/kg to X-rays exposed mice resulted in increase of animal survival rate. The IMP injection to irradiated animals, in a concentration 15 mg/kg increased the survival of mice in 10 %, and in 45 and 90 mg/kg concentrations of IMP both increased the survival in 50% as compared with control irradiated mice group. That is, IMP in a 15 mg/kg concentration does not reveal statistically significant protective properties. Increasing of IMP concentration from 45 to 95 mg/kg does not influence the survival rate. Consequently in our subsequent experiments we used IMP in a concentration 45 mg/kg.

Figure 1 shows the effect of IMP on the survival of mice treated with X-rays (7 Gy). The median of survival time of irradiated control mice was 8 days after the exposure, and a maximum survival time was 13 days. The IMP administration after X-rays exposure significantly prolonged survival

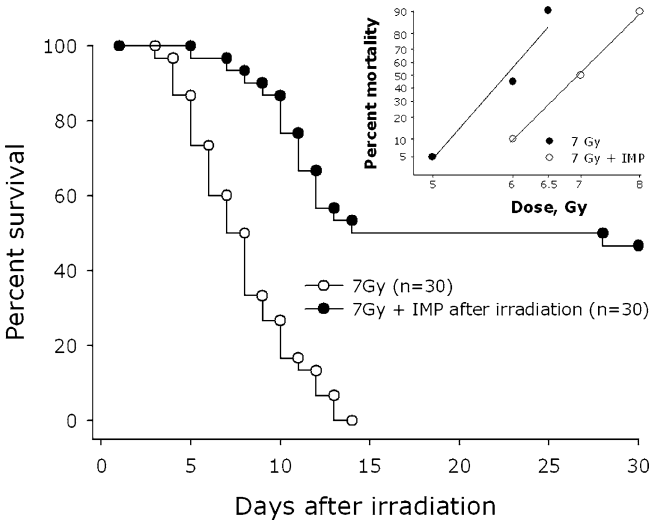

Fig. 1. Kaplan-Meier estimate of 30-day survival of X-irradiated (7 Gy) mice injected i.p. with IMP (~45 mg/kg) 15 min after irradiation. The data represent the means of three separate experiments with 10 mice per group in each experiment. Difference in survival between the irradiation control group and the IMP-treated groups was evaluated by Fisher's exact test. Inset: Radiation dose-response of IMP (~45 mg/kg) injected i.p. 15 min after 5, 6, 6.5, 7, and 8 Gy, plotted as percent mortality. For each experimental point, the data for 12–20 animals were used. Abscissa and ordinate are in the logarithmic form.

Table 1. Percentage changes in body weight (means) and the amount of food and water intake of X-irradiated (7 Gy) mice injected i.p. with IMP (~45 mg/kg) 15 min after exposure.

Treatment	Days after irradiation			
	0	5	10	30
	Change of body weight, %/Food/Water intake, g			
0 Gy	100/4.8/7.0 (10)	103/4.7/6.9 (10)	106/4.7/7.0 (10)	115/4.9/7.1 (10)
0 Gy + IMP	100/4.7/6.9 (10)	102/4.7/7.0 (10)	106/5.0/7.0 (10)	117/4.8/6.9 (10)
7 Gy	100/4.6/6.8 (10)	85/3.5/6.2 (8)	80/3.3/5.2 (2)	–
7 Gy + IMP after irradiation	100/4.8/7.0 (10)	94/4.0/6.0 (9)	92/4.4/6.1 (8)	108/5.1/7.2 (5)

Data are means for n animals; n is given in parentheses.

time of the mice. In case of IMP application a survival rate was ~45% on 30th day of the experiment compared to 100% mortality in irradiated controls. No signs of a diarrhea or an obvious rectal bleeding were observed in irradiated mice throughout the whole experiment.

To determine the dose reduction factors (DRF), we studied the dose–response relationship in the dose range of 5–8 Gy for the survival of mice within 30 days. The results are presented in Fig. 1 (Insert). It is seen that the administration of the IMP after the exposure to ionizing radiation decreased the probability of lethal outcomes. Thus, at a dose of 6 Gy, the survival of mice increased by 40% after the administration of IMP, as compared with irradiation control. The dose-response plot is convenient to use for the calculation of semi-lethal doses. For mice injected with the isotonic solution 15 min after irradiation, LD50/30 (dose inducing 50% lethality within 30 days) was 5.8 Gy. After the injection of IMP, the LD50/30 increased to 7.1. The DRF, which is calculated as the ratio of the LD50/30 doses in the presence and absence of the IMP was 1.22.

Consumption of water and food by animals as well as weight changes were also measured throughout the mice survival studies (Table 1). The consumption of food and water by intact mice increased by 15% within entire experimental period. Irradiated mice consumed food and water less by 20% if compared with intact animals. Mice injected with IMP after irradiation consumed food and water less only by 10% if compared to non-irradiated animals. Body weight in all groups of non-irradiated mice was gained, in mean, 15% over initial weight, over the time of experiment. Maximum weight loss in a group of irradiated animals was 20% and weight loss in the group of mice injected with IMP was only 8% from initial weight.

The effect of i.p. IMP administration to irradiated mice, on the leukocyte count of their peripheral blood was studied (Fig. 2). The amount of blood leukocytes in intact animals almost was not changed throughout the experiment. In unirradiated mice receiving IMP the leukocyte count was not changed (data not shown). In groups of irradiated mice an abrupt decrease of leukocyte count was observed by the second day of the experiment. By day 6 the number of white blood cells in irradiated control mice and in irradiated mice receiving IMP dropped by ~98% and ~85%, respectively if compared with the intact mice group. Then leukocyte count in irradiated control mice was not changed up to their death. In animals receiving IMP after irradiation, the amount of leukocytes began to rise starting on 14th day of the experiment and averaged 65% of its normal value by 30th day.

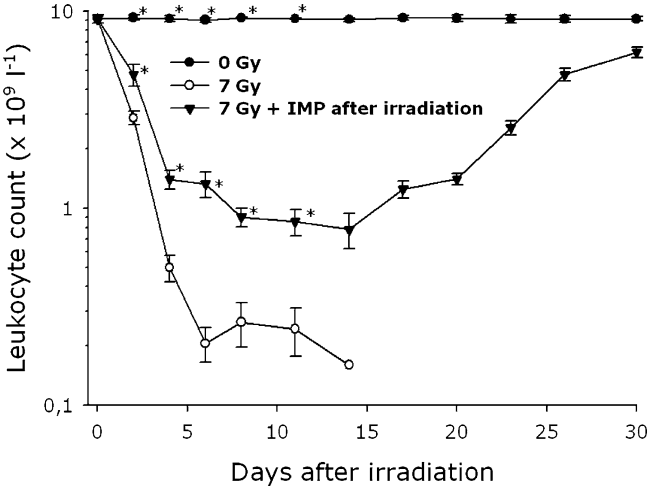

Fig. 2. Circulating leukocyte counts of whole-body 7 Gy X-irradiated mice injected i.p. with IMP (~45 mg/kg) 15 min after exposure. Data points represent means ± SEM of 2–5 animals. Statistically significant differences between irradiation control group and the other groups (Mann-Whitney U test, $p < 0.05$) are marked by asterisks.

Figure 3 shows the influence of IMP injected i.p. to mice after X-rays exposure on the granulocyte count in peripheral blood was investigated. The amount of granulocytes in non-irradiated animals was not appreciably changed throughout the entire experiment. In unirradiated mice receiving IMP the granulocyte count also was not changed (data is not shown). The drop in the number of granulocytes in irradiated mice was similar to that of leukocytes. The granulocytes count in irradiated mice by 6th day decreased by 98% com-

pared to non-irradiated control mice. In mice receiving IMP after X-ray the number of granulocytes maximally decreased only by 85% in comparison with intact mice group. Thereupon granulocyte count in IMP treated mice gradually began to rise and amounted to ~75% relative to unirradiated control.

The effect of IMP injected i.p. on the platelet count in peripheral blood of mice exposed to X-rays was studied (Fig. 4). The platelet count in unirradiated mice was not changed over the entire experiment. In unirradiated mice receiving IMP the thrombocyte count also was not changed (data not shown). In control irradiated mice the number of platelets dropped to 25% by 4th day of the experiment and to 80% by 14th day relative to the number of platelets in peripheral blood of intact mice. In irradiated animals receiving IMP after irradiation, platelet count grew slightly to 2-th day of experiment, and then tends down until the 14th day to 70% of its initial value. Upon that point, the number of platelets in this group began to increase, and it was about 90% of normal levels the on the 30th day.

Using the MN-test, we studied a role of IMP injected i.p. to mice 15 minutes after X-ray irradiation at a dose of 1.5 Gy bringing down of formation of PCE with MN in the mouse bone marrow (Table 2). The introduction of IMP to unirradiated mice had no effect on PCE with MN formation. As it is represented from Table 2, the percentage of PCE with MN after irradiation increased approximately in 15 times from 0,33% in the intact control mice to 4,90% in mice irradiated with a dose of 1,5 Gy. Administrated IMP (~15 mg/kg) to mice showed less PCE with MN in 30%

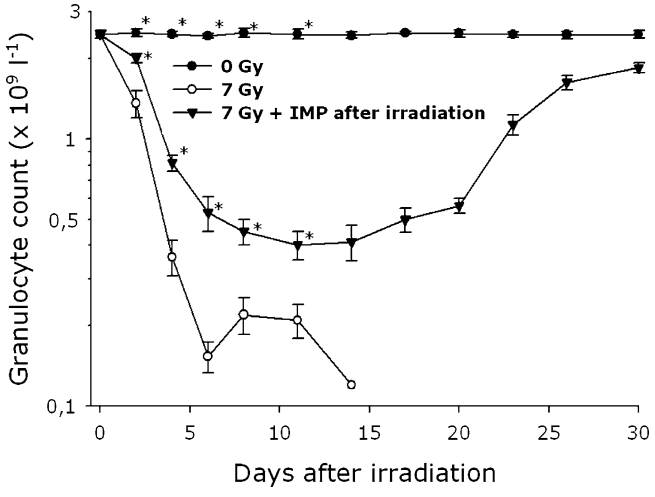

Fig. 3. Peripheral granulocyte counts of X-irradiated (7 Gy) mice injected i.p. with IMP (~45 mg/kg) 15 min after exposure. Data points represent means + SEM of 2–5 animals. Statistically significant differences between irradiation control group and the other groups (Mann-Whitney U test, $p < 0.05$) are marked by asterisks.

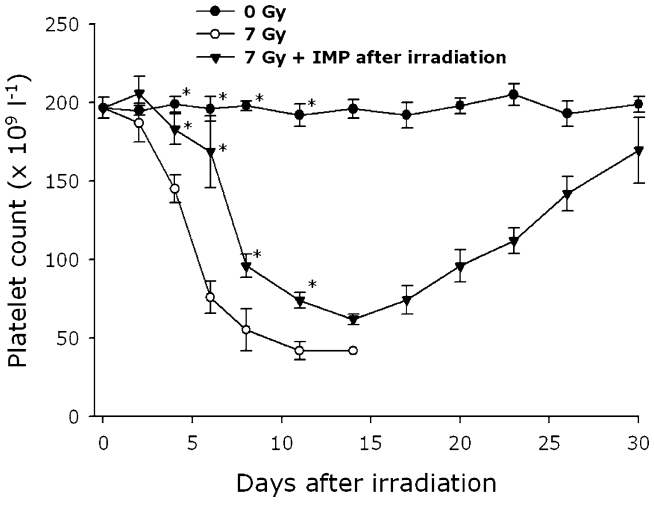

Fig. 4. Peripheral platelet counts of X-irradiated (7 Gy) mice injected i.p. with IMP (~45 mg/kg) 15 min after exposure. Data points represent means + SEM of 2–5 animals. Statistically significant differences between irradiation control group and the other groups (Mann-Whitney U test, $p < 0.05$) are marked by asterisks.

Table 2. Effect of IMP (~45 mg/kg) injected i.p. to mice 15 min after their irradiation with 1.5 Gy of X-rays on the formation of PCE with MN in the bone marrow of the animals. The data were obtained by the micronucleus test and are given as means ± SEM for specified number of animals. Asterisks indicate a significant difference at 5% level (Student's unpaired t-test) in comparison with the irradiation control.

Treatment	Number of animals	Number of PCE	Number of PCE with MN	Percentage of PCE with MN
0 Gy	5	10280	34	0.33 ± 0.06*
0 Gy + IMP	5	10236	30	0.30 ± 0.05*
1.5 Gy	5	10188	500	4.90 ± 0.43
1.5 Gy + IMP (~15 mg/kg)	5	10312	353	3.43 ± 0.46*
1.5 Gy + IMP (~45 mg/kg)	5	10241	150	1.46 ± 0.10*
1.5 Gy + IMP (~90 mg/kg)	5	10398	148	1.31 ± 0.09*

Table 3. Effect of IMP (~45 mg/kg) injected i.p. to mice 15 min after their irradiation with 7 Gy of X-rays on DNA degradation in the thymus cells of the animals. The data were obtained by the flow cytometry and are given as means ± SEM for specified number of animals. Asterisks indicate a significant difference at 5% level (Student's unpaired t-test) in comparison with the irradiation control.

Treatment	Number of animals	DNA degradation, %
0 Gy	7	11.1 ± 0.9*
0 Gy + IMP	4	10.8 ± 1.5*
7 Gy	5	69.1 ± 4.2
7 Gy + IMP after irradiation	5	48.4 ± 6.4*

comparing with control group 1.5 Gy. The introduction of IMP (~45 mg/kg) to mice after irradiation led to a decrease in the number of PCE with MN in 3 times if compare to irradiated animals. Increase of IMP concentration from 45 to 95 mg/kg had no result in any additional decline in PCE with MN count.

Table 3 presents the protective effect of IMP on DNA damage in thymus cells of mice treated with lethal dose of X rays (7 Gy). The i.p. introduction of IMP to intact mice had no a significant effect on the DNA damage level in thymus cells. In the group of irradiated mice it was observed more than sixfold increase in DNA degradation in thymus cells. In irradiated mice receiving IMP 15 minutes after irradiation the DNA damage was about 1.5 less that DNA damage in irradiated control mice group.

DISCUSSION

It is known that the main causes of post-radiation death of the irradiated animals in the dose range of 4–10 Gy are the bone marrow and gastrointestinal syndromes.[12] Previously it has been shown that the death of X-rays exposed mice (7 Gy) mainly account for the bone marrow radiation syndrome.[9] So in our experiments irradiated animals did not show any signs of diarrhea, any significantly weight changes or food and water intake (Table 1). The i.p. administration of IMP 15 min after irradiation to mice increase the leukocyte (Fig. 2) and platelet count in their peripheral blood to normal (unirradiated) status, thereby IMP reduces heaviness radiation-induced leukopenia and thrombocytopenia in mice. The main hemopoietic organs in sexually mature mammals are red bone marrow and thymus. Probably, in these organs IMP stimulates the DNA repair and, consequently, the cell recovery and proliferation. Using the MN-test and flow cytofluorometry, we demonstrated that approximately one day after IMP administration to irradiated mice decreasing of DNA damage in red bone marrow and thymus cells was observed (Table 2, 3). The IMP was demonstrated to have better protective effect if compared with GMP [6] and inosine[8] (micronucleus test (1.5 Gy): IMP > Ino > GMP; blood cells count (7 Gy) IMP > GMP > Ino; DRF: IMP (1.22) > Ino (1.15).

At present no data on specific cellular receptors for IMP. However, inosine, the product of IMP catabolism, has an ability to interact with cellular receptors. It was shown, that inosine interacts with A2a and A3 adenosine receptor subtypes,[13] which are involved in signal pathways of triggering of apoptosis, inflammation and allergic reaction.[14] Moreover, inosine increases levels of the DNA repair enzyme 8-oxoguanine-DNA-glycosylase in the alveolar epithelial type 2 cells during sub lethal hyperoxia through adenosine A3 receptors in rodent mast cells.[15] It is possible, that IMP, like inosine, also is capable of interacting with adenosine receptors.

There are two broad classes of mitigators: purine nucleoside analogs and cytokines.[4] Granulocyte-colony stimulating factor (G-CSF) is the most effective and well-studied among the growth factors. G-CSF exercises protective properties when injected to the irradiated organism during the day. G-CSF increases survival of various irradiated animals and accelerates recovery from leukopenia and thrombocytopenia.[16,17] In clinic, G-CSF is used in chemotherapy to hasten neutrophil recovery.[17] On the other hand, cytokines even at concentrations 5 μg/kg may be toxic if given systematically and can cause many side effects.[18] The radioprotective properties of IMP and cytokines are comparable, but IMP is less expensive, more stable and well-tolerated (LD50 for IMP is 3,9 g/kg and 4,8 g/kg for subcutaneous and intraperitoneal, respectively, or 15,9 g/kg-oral administration).[20] Listed above properties make the IMP extremely favorable for the application, but this compound, in contrast to G-CSF should be administered shortly after radiation exposure. For this reason, IMP, unlike the G-CSF, might be not so convenient for using in a radiological accident. However, absence of side effects and low cost makes IMP a good alternative in clinical and scientific practice.

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