Antitumor effects of Palladium-α-Lipoic Acid Complex Formulation as an Adjunct in Radiotherapy

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Abstract

Several investigations have been initiated for enhancing the antitumor effect of radiation and ameliorating its adverse effects such as the lowering of blood cell counts and DNA damage in normal cells. Compounds that enhance the antitumor activity of radiation without lowering blood cell counts or damaging DNA in normal cells can be of immense use as an adjunct in radiotherapy. The antitumor effect of Poly-MVA (2 ml/kg, p.o), with and without radiation against the Dalton's lymphoma ascites and Ehrlich's ascites carcinoma cell lines transplanted solid tumor model, was evaluated. Whole body gamma radiation exposure (2 Gy) was done with ^{60}Co. Poly-MVA enhanced the antitumor effect of radiation, when administered prior to radiation. Furthermore, Poly-MVA when administered, once daily for 2 weeks, immediately after 4 Gy irradiation, protected DNA damage in the peripheral blood. It also rendered protection against radiation-induced lowering of platelet count. The unique electronic and redox properties of palladium alpha-lipoic acid complex in Poly-MVA appear to be responsible for the exhibited effect. The results conclude that the antitumor enhancing and normal cell protective effect of Poly-MVA warrants additional studies for its potential clinical application.

*Keywords***:** Antitumor, DNA damage, Poly-MVA, Radiotherapy, Radiosensitizers, alpha-lipoic acid

INTRODUCTION

Despite the use of chemotherapy and radiotherapy, cancer eradication remains a major challenge to mankind. Radiotherapy, using external beam such as X-rays, gamma rays, and charged particles to shrink tumors or kill cancer cells, can be used as an adjuvant or neoadjuvant to surgery and chemotherapy for a majority of the cancer patients. [1] It may be used to treat localized solid tumors, such as cancers of the skin, tongue, larynx, brain, breast, lung, prostate or uterine cervix. Most of the radiation treatments apply low doses of ionizing radiation (IR) (1.8–2 Gy fractions over the course of 4–8 weeks) to local regions of the body, which can either damage DNA directly or through free radicals generated within the cells that, in turn, damage the DNA. [2] Radiosurgery/stereotactic body radiation therapy uses high doses of radiation (15–20 Gy) to many cancer types including non-small-cell lung cancer, prostate cancer, renal cell carcinoma and hepatocellular carcinoma with limited toxicity to normal tissue. [3] Similarly, intensity modulated radiation therapy uses non-uniform, computer-optimized radiation fields to deliver a high dose of radiation to the tumor. [4] Regardless of the type of radiotherapy, radiation exposure causes free radicals mediated cytotoxicity to normal cells that has to be addressed. Furthermore, this modality alone seldom achieves a satisfactory therapeutic outcome in many cases. Hence, limiting toxicity of IR without compromising its antitumor efficacy remains the major challenge to clinicians. Agents that can attenuate the radiation-induced toxicity, while enhancing the antitumor effect, would be a welcome addition to cancer treatment.

Poly-MVA is a palladium lipoic acid complex formulation, available commercially as a dietary supplement. [5] Its major active ingredient is a palladium–lipoic

acid polymer that exists as a trimer of palladium-lipoic acid joined to thiamine. This organo-metallic complex demonstrates enhanced water and lipid solubility, and a potent redox activity upon electrochemical impedance analysis. "MVA" stands for minerals, vitamins, and amino acids.[5] The minerals include molybdenum, rhodium, and ruthenium, whereas the B complex vitamins are B_1, B_2, and B_{12}. Amino acid derivatives, N-acetyl cysteine (NAC) and formyl methionine, are also included in this liquid blend.[5] Recently, we reported Poly-MVA as an effective agent to protect against the age-linked decline of antioxidant status in myocardial and cerebral mitochondria, as well as being able to maintain energy production in the aged rat mitochondria (brain and heart).[6,7] Poly-MVA also exhibited *in vitro* free radical scavenging activity.[8] Efforts have been initiated to develop novel non-platinum-based antitumor agents to improve clinical effectiveness, to reduce general toxicity as well as to broaden the spectrum of activity. Palladium, like platinum, belongs to the same transition metal group. Research continues on substances, such as fluorouracil and cisplatin, to indentify new radiosensitizing substances, in order to make tumors more specific and sensitive to low dose radiation without affecting normal tissues. Since the antitumor effects of Poly-MVA, or Poly-MVA with radiation, have not yet been reported, we examined it as an adjunct to radiation in these studies.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (20 ± 2g) were purchased from Small Animal Breeding Center, Kerala Agricultural University, Mannuthy, Thrissur, Kerala, India and kept for a week under environmentally controlled conditions with free access to standard food and water

ad libitum. The animal experiments were conducted according to the rules and regulations of Institutional Animal Ethics Committee, Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India which followed the CPCSCA, Govt. India guidelines

Cell lines

Ehrlich's ascites carcinoma (EAC) and Daltons lymphoma ascites (DLA) cell lines were obtained from Cancer Institute, Adayar, Chennai, India. The cells were maintained in mice by intraperitoneal inoculation of $1x10^6$ viable cells.

The palladium lipoic acid formulation was obtained as a gift from Garnett McKeen Laboratory, Inc., USA.

Irradiation

Irradiation was carried out using a ^{60}Co-Theratron Phoenix Teletherapy Unit (Atomic Energy Ltd., Ottawa, Canada) at a dose rate of 1.88 Gy per minute.

Antitumor effect of Poly-MVA with or without radiation in animals bearing EAC transplanted solid tumor

Albino mice were injected subcutaneously with 1-2 x 10^5 live cells of EAC in PBS in the hind limb. After ~ 10 days, animals with palpable tumor were divided into 4 groups of 6 animals each. Animals in Group I were kept as untreated control; Group II animals treated with Poly-MVA (2 ml/kg, p.o) once daily for 2 weeks; Group III animals treated with 2 Gy radiations once a week for 2 weeks; Group IV animals treated with Poly-MVA (2 ml/kg, p.o) daily for 2 weeks and 2 Gy whole body gamma radiations from ^{60}Co once a week for 2 weeks, 1 hr after the Poly-MVA administration. Tumor volume was measured once weekly. Animals were sacrificed 24 hrs after the last dose of

irradiation/Poly-MVA treatment. The tumor was extirpated, weight measured and percent inhibition was calculated using the formula [C-T]/C where C is the tumor volume or tumor weight of the control group, and T is that of the treated group. [9]

Antitumor effect of Poly-MVA with or without radiation in animals bearing DLA transplanted solid tumor

Albino mice were injected subcutaneously with 1-2 x 10^5 live cells of DLA in PBS in the hind limb. After ~ 10 days, animals with palpable tumor size were divided into 4 groups of 6 animals each and treated as described above. Tumor volumes were measured before the treatment, on the 17^{th} day (1 week after the treatment) and again after the commencement of treatment. Animals were sacrificed 24 hrs after the last dose of irradiation/Poly-MVA treatment and EDTA-blood samples were collected. Platelet counts and single cell gel electrophoresis (Comet) assay were performed in the blood to detect the DNA damage. Fifty cells on each slide were selected at random sites for the quantification of the single stranded-DNA breaks using the 'CASP' software system. The DNA damage was compared with that of the control group.

Effect of Poly-MVA against radiation induced DNA damage

Albino mice were divided into 3 groups of 6 animals each. Group I was kept as an untreated control (normal); Group II animals were treated with a single dose of 4 Gy radiation and kept as a radiation control. Group III animals were treated with Poly-MVA (2 ml/kg, p.o) immediately after the exposure of 4 Gy radiation. The mice were sacrificed 24 hrs after the irradiation and EDTA-blood was collected. Single cell gel electrophoresis (Comet) assay was performed. Fifty cells on each slide were selected at random sites for

the quantification of the single stranded-DNA breaks using the 'CASP' software system. The DNA damage was compared with that of the control groups.

Statistical analysis

All data were represented as mean ± SD. The mean values were statistically analyzed using one-way analysis of variance (ANOVA) (using the Graph Pad Instat software package, CA, USA). The significant differences between the groups were further analyzed by Bonferroni's t-test and Dunnett multiple comparison test for treated verses the control group. p value of less than 0.05 was considered significant.

RESULTS

Antitumor effect of Poly-MVA with or without radiation against EAC-induced tumor

Administration of Poly-MVA (2 ml/kg, once daily for 2 weeks) in combination with whole body γ-radiation (2 Gy, once per week for 2 weeks) showed significant antitumor effects when compared to the control (tumor bearing animal without treatment) (Table 1). The tumor volumes were 0.39 ± 0.018 cc and 0.37 ± 0.013 cc in the groups treated with radiation alone and Poly-MVA + radiation, respectively. The control group showed final tumor volume of 1.02 ± 0.07 cc. While the antitumor effects were found to be significant ($p<0.01$), with respect to the control group, no statistically significant difference could be observed between the treated groups. Percent inhibition according to the tumor weight were 55%, 61% and 65% for the Poly-MVA, radiation alone and Poly-MVA + radiation treated groups, respectively (Fig. 1)

Antitumor effect of Poly-MVA with or without radiation against DLA-induced tumor

Effect of Poly-MVA on the tumor volume is given in Table 2. Administration of Poly-MVA once daily for 14 days, or radiation alone, could effectively inhibit the tumor growth when compared to that of the control group. However, the antitumor effect was better for the combination treatment as is evident in the tumor volume of Poly-MVA plus radiation treated group. Similarly, the tumor weight was also significantly reduced in all the treated groups with the maximum decrease observed in the Poly-MVA + radiation treated group (Fig. 2), which demonstrated an 80% inhibition ($p<0.01$).

No statistically significant effect was observed in the DNA damage in any of the treated groups (Table 3). A slight elevation was observed in the mean value (statistically non-significant) of tail length in the Poly-MVA, radiation alone and also in the Poly-MVA plus radiation treated groups.

Platelet count decreased in the radiation alone treated tumor bearing mice (Fig. 3). Poly-MVA administration prior to the radiation increased the platelet count but was found to be statistically non-significant with respect to the radiation alone treated groups.

Effect of Poly-MVA on DNA damage

The results of the DNA damage in the presence and absence of Poly-MVA are given in Table 4. Following 4 Gy of irradiation, damage to DNA in the cells of the peripheral blood was found to be highest in the control group, when analyzed 24 hrs after the irradiation (Fig.4). DNA damage was ameliorated significantly by Poly-MVA ($p<0.001$).

DISCUSSION

In the present study, Poly-MVA effectively ameliorated radiation-induced toxicity when administered immediately after IR exposure. IRs are involved with several health hazards including cancer and the risk of birth defects. When water, the most abundant

intracellular material, is exposed to IR decomposition occurs generating a variety of reactive oxygen species (ROS), such as superoxide radical and hydroxyl radical. These ROS can contribute to radiation injury in cells. The response of cells towards the IR exposure differs quantitatively and qualitatively, according to the absorbed dose and the cell type. Lymphocytes are particularly susceptible to DNA damage. However, we could not find any change in the DNA damage, in the 2 Gy irradiated solid tumor bearing animals. This may probably due to very low dose of radiation. Previous studies have found that increasing the dose of radiation (significant at 4 Gy) and length of the post irradiation period will result in the decline of peripheral lymphocytes. Whole-body irradiation leads to a decreased concentration of all cellular elements in the blood due to the direct destruction of mature circulating cells, loss of cells from the circulation by hemorrhage or leakage through capillary walls and reduced cell production.[10] Stem cells and early progenitor cells are the most radiosensitive of all.[11]

Results of the present study reveal that Poly-MVA, with low dose radiation, is effective at preventing tumor growth. A solid tumor model selected in this study was aimed to determine the radio-sensitizing effect of Poly-MVA. The breakdown of microvasculature in solid tumors produces an inadequate supply of cellular nutrition that causes hypoxic foci, with surrounding normal stroma. These radio-resistant hypoxic cell populations attribute to the failure of conventional radiotherapy as well as facilitate the local recurrences of solid tumors. Increasing the radiation dose to these solid tumors may effectively increase the local cure/control rates. However, an increase in dose would most likely damage the peripheral normal stroma more than the hypoxic neoplastic cells. Hence, the use of chemical radiosensitizers to specifically modify the tumor sensitivity to

conventional radiotherapy becomes an attractive alternative. Many clinicians and radiotherapists are examining the use of radiosensitizers in patients.[12]

Additionally, an immediate side effect of radiation exposure is DNA damage in peripheral blood cells. Damage to spleen and bone marrow cells is a latter event. Our results demonstrate a significantly higher amount of DNA damage to the peripheral blood of the radiation control group of animals compared to the normal. The immediate administration of Poly-MVA, after the irradiation, was found to effectively protect DNA damage in these cells, whereas the result of the previous study demonstrated that when Poly-MVA was administered, prior to a lethal dose of 8 Gy whole body gamma-radiation, spleenocyte and bone marrow cell DNA was protected.[13] As a free radicals scavenger, it can spare the endogenous antioxidant enzyme system and thus improve the protection against radiation-induced damage. The radiation-induced late effects are caused, in part, by chronic oxidative stress. Increased production of ROS leads to oxidation of DNA, as well as activation of pro-inflammatory factors.[14] Cells have various enzymatic and non-enzymatic defense systems to control ROS species. However, a certain fraction that escape the cellular defense may cause permanent or transient damage to nucleic acids within the cells, leading to events such as DNA strand breakage and disruption of Ca^{++} metabolism. Electrochemical analysis of Poly-MVA demonstrated that palladium alpha-lipoic acid complex oscillates between an oxidized and reduced form as it accepts unpaired electrons and donates them to enzymatic sites. [16] This unique electronic and redox property of the palladium alpha-lipoic acid complex, in Poly-MVA, appears to be a key to its physiological effectiveness as a radioprotector in normal cells. In cancer cells it acts as an oxidizer of membrane fatty acids, serving as a redox shunt to

provide a continuous river of reducing equivalents, which appears to be its mechanisms of action as a radiosensitizer. [16] Being soluble in water and lipid solvents, Poly-MVA can render protection from free radicals to both normal cell membrane, molecules in the cytosol, and DNA.

The low oxygen levels in solid tumors are associated with a poor response to radiotherapy. Hence, the use of radiosensitizers is intended to enhance tumor cell killing while having much less effect on normal tissues.[17] Many natural and synthetic compounds have been tested as radiosensitizers over the last decades. Some drugs target different physiological characteristics of the tumor, particularly the hypoxia associated with radioresistance. Platinum chemotherapeutic drugs, such as cisplatin, oxaliplatin and carboplatin, sensitize tumors to radiation by inhibiting DNA repair.[18] Cisplatin is used in conjunction with IR to treat various types of cancer, including cervical carcinoma, head and neck cancers. [19,20] However, radiosensitizing properties of the platinum analogs have not been widely used for their sensitizing effects, in part, because of the risk of enhancing radiation-induced damage to normal tissues. A combination of synthetic antioxidants such as, amifostine, dexrazoxane, and mesna with external beam radiation therapy can significantly mitigate the adverse effects. [21] Poly-MVA is found to target cancer cells, which operate using anaerobic metabolic conditions, and kill these cells in part through its ability to change the cells' electrochemical circuitry. [22] Palladium like platinum is an electron-affinity agent, and is expected to act as a radiosensitizer. When compared to platinum drugs, palladium (II) compounds lack cross-resistance.[23,24] Platinum compounds have significant disadvantages including poor water solubility and serious side effects. [24] Some palladium complexes show significant antitumor activity,

reduce resistance of tumor cells as well as lower side effects. [24] The results of this study indicate that Poly-MVA exhibited a marked antitumor effect. However, the antitumor effect of Poly-MVA was significantly improved when given in conjunction with radiation. The mechanism of this synergy remains largely undefined.

The antitumor activity of Poly-MVA was enhanced by the addition of radiation, as evident from the decreased tumor volume in the radiation plus Poly-MVA treated group of animals. No significant DNA damage at 2 Gy radiation was observed in this experiment. The platelet count was decreased significantly in the radiation alone treated group, but this was ameliorated by the Poly-MVA. Hence, this study concludes that Poly-MVA shows antitumor activity and its effect is enhanced with a mild dose of radiation. At the same time, Poly-MVA significantly attenuates the radiation side effect of a decreased platelet count.

The presence of the free radical scavenger, lipoic acid and the addition of an alternative energy source, palladium, suggests the consideration of palladium lipoic acid in the treatment of various cancers. [25, 26] B complex vitamins in the Poly-MVA formulation can render protection to healthy cells and are able to prevent the adverse effects of radiation such as skin and gastrointestinal mucosa reactions. N-acetyl cysteine (NAC) is particularly important to prevent radiation induced cytotoxicity and genotoxicity of bone marrow. [27] Furthermore, NAC can reduce inflammation, alleviates oxidative stress, improves energy status, and ameliorates tissue damage in the intestine. [28,29] Recently, ruthenium, one of the minerals present in the Poly-MVA, has demonstrated antitumor and anti-metastatic properties with low systemic toxicity in

animal models. [30] Anticancer properties of rhodium, another mineral found in the Poly-MVA formulation, have also been reported using cancer cell lines in *ex vivo.*[31]

The radioprophylactic effects of Poly-MVA against acute doses of gamma radiation were further supported by its effect in elevating the cellular reduced glutathione level, catalase and superoxide dismutase activities in liver. [32] Oxygen Radical Absorbing Capacity (ORAC) study has found that Poly-MVA is an effective antioxidant due to the electron transferring potential of Pd. The ORAC values are 5.65, 1.6, 1.4, 1.12 and 1.0 for Poly-MVA, Vitamin A, α-lipoic acid, vitamin C, and vitamin E, respectively. Mutagenic effect by *in vitro* Ames' test showed no mutagenic effect by this formulation.[33] Toxicological studies indicated that the LD_{50} of Poly-MVA exceeded 5000 mg/kg. This tested dose corresponded to ~10 ml/day in a 70 kg human (3.36mg/kg). Administration of 0.05 ml/kg of Poly-MVA (which is equivalent to 0.38 mg complexed alpha-lipoic acid/kg, p.o), once daily for 30 days, was significantly effective to enhance the Krebs cycle dehydrogenases, and mitochondrial electron transport chain complexes.[34] Poly-MVA was also effective as a hypoglycemic agent against the alloxan-induced diabetic model, as well as in the oral glucose tolerance test in rats.[8] The energy providing property of Poly-MVA could also be useful to alleviate the fatigue that most commonly occurs during cancer therapy. Overall, the activities of Poly-MVA may suggest its role as a potential adjunct in radiotherapy.

Conclusion

The results concluded that the Poly-MVA protected DNA damage in the peripheral blood when administered immediately after the radiation exposure. Poly-MVA enhanced its own anti-tumor effects when administered with a mild dose of radiation. Furthermore, it

also protected the decline of blood cell count and DNA damage consequent to radiation treatment. Due to its potent redox ability, Poly-MVA appears to provide a cellular electron source that can be utilized to potentiate radiotherapy and alleviate its side effects.

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Legend to figures

Fig. 1. Effect of Poly-MVA with or without radiation against Ehrlich's ascites carcinoma cell line induced tumor weight Values are mean ± SD, n = 6, **p <0.01 (Dunnett multiple comparison test) significantly different from the control group

Fig. 2. Effect of Poly-MVA with or without radiation against Dalton's lymphoma ascites cell line induced tumor weight Values are mean ± SD, n = 6, **p <0.01 (Dunnett multiple comparison test) significantly different from the control group

Fig. 3. Effect of Poly-MVA against radiation induced platelet count in mice bearing solid tumor induced by Dalton's lymphoma ascites cell line. *p < 0.05 significantly and NS non-significantly (Dunnett multiple comparison test) different from the control group.

Fig. 4. Photograph of DNA damage after the exposure of 4 Gy radiation. The mice were sacrificed 24 hrs after the irradiation, EDTA-blood was collected and single cell gel electrophoresis (Comet) assay was performed. A) Normal B) Poly-MVA (2 ml/kg) +4 Gy radiation and C) Control 4 Gy radiation alone.

Table1. Effect of Poly-MVA with or without radiation against the Ehrlich's ascites carcinoma cell line induced tumor

Groups	Treatments	Tumor volume prior to the treatment (cm^3)	Tumor volume 1 week after starting the treatment (cm^3)	Final tumor volume (2 weeks after starting the treatment) (cm^3)
Control	--	0.33 ± 0.05	0.64 ± 0.06	1.02 ± 0.07
Poly-MVA	2 ml/kg, p.o	0.31 ± 0.09^{NS}	0.39 ± 0.10*	0.41 ± 0.01* (59%)
Radiation	2 Gy	0.33 ± 0.05 ^{NS}	0.42 ± 0.13*	0.39 ± 0.018* (62%)
Poly-MVA + Radiation	2 ml/kg, p.o + 2Gy	0.32 ± 0.10 ^{NS}	0.42 ± 0.09*	0.37 ± 0.013* (64%)

Values are mean ± SD, n = 6; Value in parenthesis indicate percent inhibition
*P<0.05(Dunnett multiple comparison test) significantly and ^{NS} p>0.05 non-significantly different from the control group.

Table 2. Effect of Poly-MVA with or without radiation against the volume of Dalton's lymphoma ascites induced solid tumor

Groups/Treatments	Tumor volume prior to the treatment (cm^3)	Tumor volume 1 week after starting the treatment (cm^3)	Final tumor volume (2 weeks after starting the treatment) (cm^3)
Control	0.44 ± 0.03	1.82 ± 0.18	2.05 ± 0.31
Poly-MVA(2 ml/kg, p.o)	0.44 ± 0.02 ^{NS}	0.61 ± 0.11**	0.75 ± 0.21** (63%)
Radiation (2 Gy)	0.43 ± 0.02 ^{NS}	0.67 ± 0.20**	0.74 ± 0.20** (64%)
Poly-MVA (2 ml/kg, p.o) + Radiation (2Gy)	0.42 ± 0.02 ^{NS}	0.39 ± 0.17**	0.42 ± 0.13** (80%)

Values are mean ± SD, n = 6; Value in parenthesis indicate percent inhibition
** $p < 0.01$ (Dunnett multiple comparison test) significantly and ^{NS} $p>0.05$ non-significantly different from the control group.

Table 3. Effect of Poly-MVA with or without 2 Gy radiation on the DNA damage in Dalton's lymphoma ascites induced solid tumor

Groups/ Treatments	Tail DNA%	Tail length (µm)	Tail moment	Olive tail moment
Control	0.30 ± 0.21	7.36 ± 3.58	0.03 ± 0.02	0.14 ± 0.08
Poly-MVA (2 ml/kg, p.o)	0.44 ± 0.25	7.90 ± 2.06	0.04 ± 0.02	0.15 ± 0.08
Poly-MVA (2 ml/kg, p.o)+ Radiation	0.47 ± 0.19	7.84 ± 3.18	0.04 ± 0.01	0.18 ± 0.09
Radiation	0.45 ± 0.28	8.58 ± 3.61	0.05 ± 0.03	0.19 ± 0.05

Values are mean ± SD, n = 6
All values are statistically (Dunnett test) non-significant ($p > 0.05$) with respect to the control group.

Table 4. Effect of Poly-MVA (2 ml/kg, p.o) against DNA damage in the peripheral blood sample of animals irradiated with 4 Gy radiation.

Parameters	Normal	Control (4 Gy)	Radiation + Poly MVA
Tail DNA%	3.32 ± 0.12	27.11 ± 6.50[a]	0.27 ± 0.18***
Tail length (µm)	5.30 ± 0.64	69.49 ± 15.05 [a]	4.91 ± 1.77***
Tail moment	0.74 ± 0.07	19.26 ± 7.29 [a]	0.02 ± 0.01***
Olive tail moment	0.85 ± 0.05	14.27 ± 4.34 [a]	0.09 ± 0.06***

Values are mean ± S.D, n = 6, [a] $p < 0.001$ (Bonferroni multiple comparison test) significantly different from the normal group and *** $p < 0.001$ (Dunnett test) significantly different from the control group.

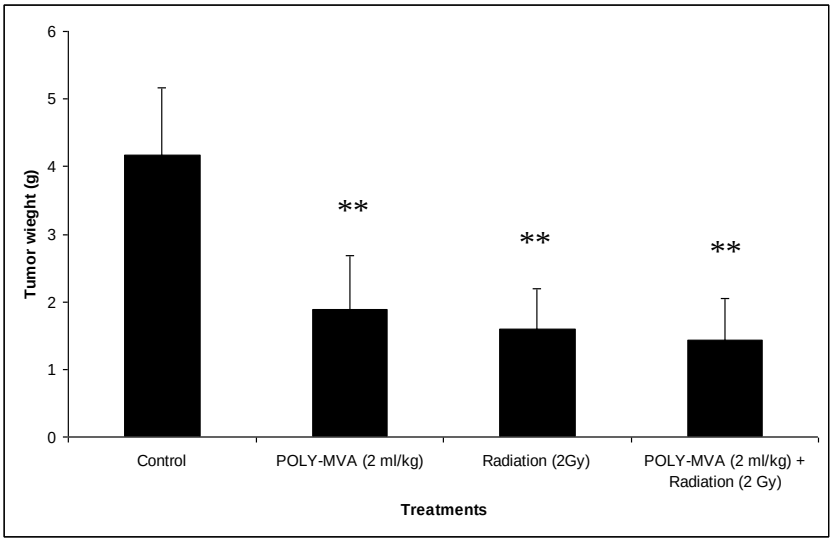

Figure 1.

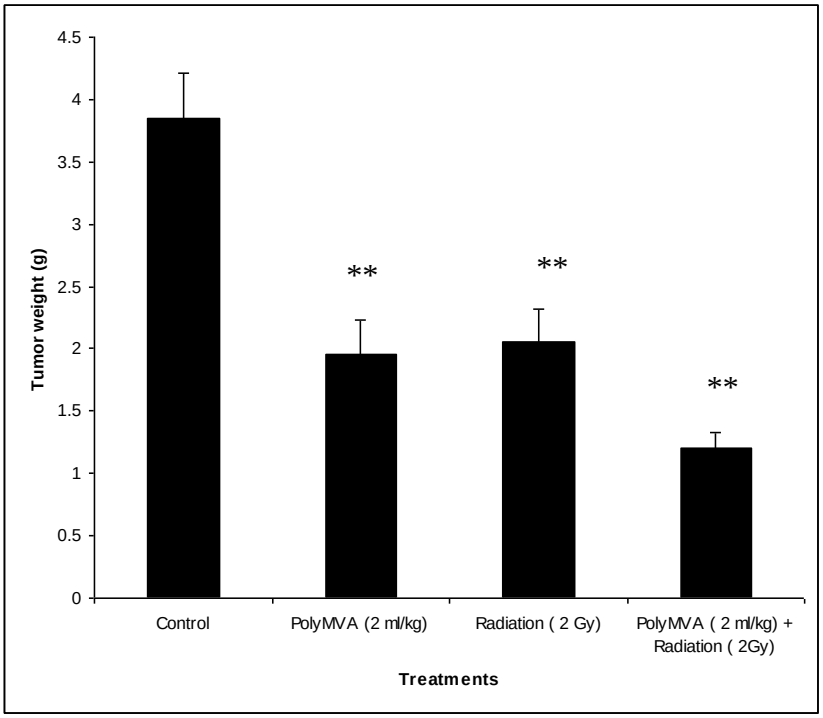

Figure 2

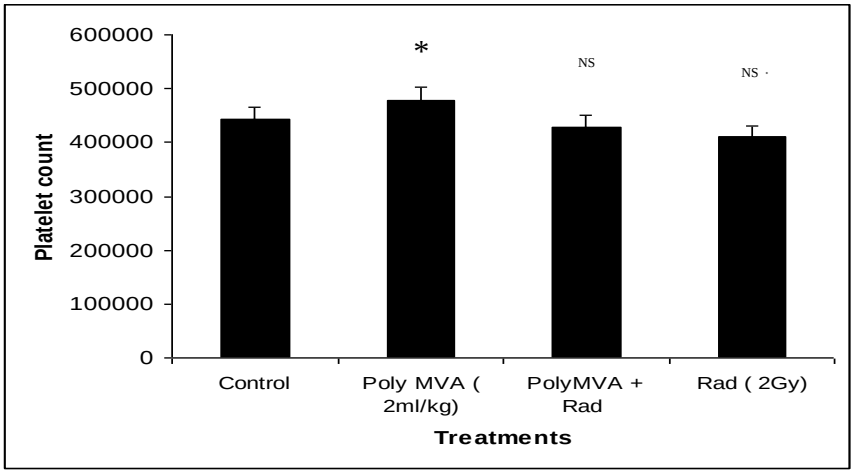
600000
500000
400000
300000
200000
100000
0
Platelet count
*
NS
NS
Control
Poly MVA (2ml/kg)
PolyMVA + Rad
Rad (2Gy)
Treatments

Figure. 3