In vivo study of radioprotective effect of NO-synthase inhibitors and acetyl-L-carnitine

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Abstract

This study investigated the protective effect of two nitric oxide synthase inhibitors N(omega)-Nitro-L-arginine methyl ester (L-NAME, 100 mg/kg i.p.) and aminoguanidine (AG, 400 mg/kg i.p.), and an antioxidant acetyl-L-carnitine (ALC, 250 mg/kg i.p., once daily for five days) against radiation-induced damage in Wistar rats. Blood samples were collected 6 hrs after whole-body irradiation with 8 Gy. Plasma concentrations of nitrite+nitrate (NOx) and malondialdehyde (MDA) were measured by high-performance liquid chromatography. A single injection of L-NAME one hour before exposure effectively prevented the radiation-induced elevation of plasma NO_x and it reduced 2.6-fold the risk for death during the subsequent 30-day period. Pretreatment with ALC prevented the radiation-induced increase in plasma MDA and it had similar effect on mortality as L-NAME did. Presumably due to its short half-life, the partially iNOS-selective inhibitor and antioxidant AG given in a single dose before exposure did not attenuate MDA and NOx and it failed to significantly improve the 30-day survival. In conclusion, pretreatment with both the nonspecific NOS inhibitor L-NAME and the antioxidant ALC markedly reduce mortality to radiation sickness in rats. The radioprotective effect may be directly related to effective attenuation of the radiation-induced elevation of NO production by L-NAME and of oxidative stress by ALC.

Key words: acetyl-L-carnitine; aminoguanidine; ionizing radiation; N(omega)-Nitro-L-arginine methyl ester

Introduction

Ionizing radiation-induced injury is mediated either directly or indirectly, i.e. via the radiolytic products of water. In particular, free radicals H• and •OH lead to the production of toxic reactive oxygen species (ROS) such as superoxide radical ($•O_2^-$) and hydrogen peroxide (H_2O_2). Moreover, nitric oxide (NO), a gaseous reactive radical, is formed in higher amounts by NO synthases (NOS) from L-arginine in response to ionizing radiation. The reaction of $•O_2^-$ with •NO generates peroxynitrite ($ONOO^-$), a powerful oxidant (Haghdoost 2005). ROS as well as $ONOO^-$ and other reactive nitrogen species (RNS) attack molecules of biological importance, especially DNA, lipids and proteins (Arterbery et al. 1994). In addition, some theories suggest that ROS may function as initiators and NO

and other RNS as effectors activating cytosolic signal transduction pathways in response to ionizing radiation (Mikkelsen and Wardman 2003).

There exist three isoforms of NOS: constitutively expressed neuronal (nNOS) and endothelial (eNOS) isoforms and the inducible NOS (iNOS) which generates comparatively larger amounts of NO, e.g. in response to inflammation (Ricciardolo 2004). The levels of plasma nitrite (NO_2^-) and nitrate (NO_3^-), the stable and predominant metabolites of NO, can be used as biomarkers for NO synthesis *in vivo* (Tsikas et al. 2006). Our previous study described the changes in L-arginine-NO metabolic pathways which occur within a 24-hour period after whole-body gamma irradiation of rats with the range of 2-50 Gy. A dose-dependent increase of the sum of plasma NO_2^- and NO_3^- concentrations (NO_x) was detected at 6 hr following irradiation with the maximum NO_x level 4-fold above the controls after 10 Gy. On the contrary, radiation-induced generation of NO was modest or absent in the airways and lungs (Babicova et al. 2011). Other investigators were able to demonstrate that ionizing radiation of rodents increases the expression of iNOS mRNA and protein and augments the concentrations of NO_2^-, NO_3^- and nitrotyrosine within the first 24 h (Huang et al. 2009; Clarençon et al. 1999) as well as during later periods of several weeks following exposure (Tsuji et al. 2000; Nozaki et al. 1997; Giaid et al. 2003).

The primary cause of mortality to radiation sickness are hematopoietic and gastrointestinal syndromes associated with sepsis due to acquired immunodeficiency state and increased translocation of bacteria across the disrupted intestinal barrier. This is further complicated by bleeding and gastrointestinal loss of water and electrolytes (Weiss and Landauer 2003). Published studies support the role for NO as a negative regulator of hematopoietic stem and progenitor cells (Michurina et al. 2004; Epperlya et al. 2007). Inhibition of NOS activity in mice by isoform-nonselective inhibitors N(omega)-nitro-L-arginine methyl ester or 2-ethyl-2-thiopseudourea hydrobromide) increases the number of stem and progenitor cells in the bone marrow and of neutrophils in the blood of intact animals or after irradiation with lethal doses and bone marrow transplantation (Michurina et al. 2004). A large body of evidence also suggests that NO is an important mediator involved in intestinal injury and breakdown of gut barrier function. The iNOS-knockout mice are resistant to loss of gut barrier function and intestinal injury induced by diet or ischemia-reperfusion (Deitch et al. 2002; Suzuki et al. 2000). In vitro experiments as well as in vivo studies using various models document upregulation of iNOS, overproduction of NO and an increased nitrossative stress which correspond with enterocyte

apoptosis (Nadler and Ford 2000). More recently, an NO-mediated increase in paracellular permeability and bacterial translocation into the blood stream was ascribed to disruptions in tight junctions, mediated through a pathway that involved myosin light chain, myosin phosphatase target subunit 1, and protein kinase C [zeta] (Wu et al. 2011). In these models, inhibition of iNOS activity with isoform-nonselective or iNOS-selective inhibitors had beneficial effects (Takizawa et al. 2011; Sorrells et al. 1996; Mishima et al. 1998; Unno et al. 1997).

For a long time, small molecules or enzymes with antioxidant characteristics have been investigated for their capacity to prevent radiation-induced injury both with regard to reduction of immediate toxicity and for amelioration of indirect radiation effects including long-term oxidative damage (Okunieff et al. 2008). L-carnitine and its esters (acylcarnitines) have an important role in cellular energy production (Peluso et al. 2000). Due to their antioxidant and radical scavenging effects, the compounds were shown to be beneficial in various diseases characterized by an increased oxidative stress such as heart and renal failure. A protective effect has also been demonstrated against ischemia–reperfusion injury (Sener et al. 2004; Vescovo et al. 2002; Packer et al. 1991). The radioprotective effect of L-carnitine against local irradiation has been documented on several tissues and organs including bone marrow, small intestine, kidney, lens, retina, brain, cochlea, oral mucosa and salivary glands of rats or guinea pigs. In most of these studies, a once daily dosing of L-carnitine was started on the day of radiation exposure and continued over several days thereafter (Kocer et al. 2007; Uçüncü et al. 2006; Altas et al. 2006; Caloglu et al. 2012).

The aim of this study was to evaluate the radioprotective effects of a non-selective NOS inhibitor N(omega)-nitro-L-arginine methyl ester (L-NAME), of L-carnitine ester acetyl-L-carnitine (ALC) with antioxidant effects, and of aminoguanidine (AG), a partially iNOS-selective inhibitor (Misko et al. 1993) which simultaneously acts as an antioxidant (Courderot-Masuyer et al. 1999). In a 30-day radiation survival study, Wistar rats were injected with L-NAME or AG one hour before exposure, or, they were pretreated with ALC once daily over 5 days with the last dose one hour before the whole-body irradiation with 8 Gy. Moreover, malondialdehyde (MDA) and NOx were measured in the plasma collected at 6 h post-irradiation as biomarkers of lipid peroxidation and NO production.

Materials and methods

Animals

Specific pathogen free, female Wistar rats (Velaz Ltd., Prague, Czech Republic), weighing 191-260g, were used during the experiments. Animals were fed with standard feed ST-1 (Velaz Ltd, Prague, Czech Republic) and water *ad libitum*. The animals were kept in an air- and humidity-controlled room (22 ± 2°C and 50 ± 10% relative humidity) with a 12hour light/dark cycle (7^{00} to 19^{00}). All procedures were approved by the Ethical Committee of the Faculty of Military Health Sciences Hradec Kralove (Czech Republic).

Chemicals

O-Acetyl-L-carnitine hydrochloride, N(omega)-Nitro-L-arginine methyl ester hydrochloride, Aminoguanidine hydrochloride, Phosphate-buffered saline (PBS), Malondialdehyde tetrabutylammonium salt, 2,4-dinitrophenylhydrazine, 2,3-diaminonaphtalene, nitrate reductase from Aspergillus niger, glucose-6-phosphate dehydrogenase, glucose-6-phosphate and NADPH were purchased from Sigma-Aldrich (Prague, Czech Republic).

Gamma irradiation

Whole-body irradiation at the dose of 8 Gy was performed with the help of a ^{60}Co gamma-ray source (Chirana, Prague, Czech Republic) using a dose-rate of 1 Gy/min.

Experimental design

The rats were divided into eight groups of 5-9 animals each: control PBS (C-PBS), irradiated PBS (I-PBS), control L-NAME (C-L-NAME), irradiated L-NAME (I-L-NAME), control ALC (C-ALC), irradiated ALC (I-ALC), control AG (C-AG) and irradiated AG (I-AG). L-NAME, ALC and AG were dissolved in PBS and injected intraperitoneally with100 mg/kg (L-NAME), 250 mg/kg (ALC) and 400 mg/kg (AG). Control rats were administered equal volumes of phosphate-buffered saline (PBS). L-NAME, AG and PBS were applied as a single dose one hour before irradiation. ALC was administered for 5 days with the last dose one hour before irradiation. In additional irradiated groups, mortality was recorded over 30 days after exposure. In additional irradiated groups (I-PBS, n=26 rats; I-L-NAME, n=20 rats; I-AG, n=9 rats), mortality was recorded over 30 days after exposure.

Sample collection and analysis

Based on our previous experience and the results of others, biomarkers of NO production and lipid peroxidation were investigated in the plasma collected at 6 hr following irradiation (Babicova et al. 2011, Sener et al. 2006). Rats were anaesthetized with 50 mg/kg i.p. sodium pentobarbital (Sigma Aldrich, Prague, Czech Republic). Blood was collected from abdominal aorta into a 9-mL S-monovette

K3EDTA (Sarstedt Ltd, Prague, Czech Republic). Rats were sacrificed by bleeding-out. The whole blood was centrifuged at 2000 RCF for 10 minutes at -4°C, and the plasma was separated and stored before analysis.

The sum of nitrite NO_2^- and nitrate NO_3^- concentrations (NOx) was determined using a high-performance liquid chromatography method described previously (Woitzik et al. 2001). The method detects NO_2^- after derivatization with 2, 3-diaminonaphtalene. Prior to the analysis, NO_3^- in the plasma samples was reduced to NO_2^- by the enzyme nitrate reductase from *Aspergillus niger* (Sigma Aldrich, Prague, Czech Republic).

The concentration of plasma MDA was assayed by the HPLC published by Pilz (Pilz et al. 2000), after minor modifications. The principle of this reaction is derivatization of MDA with 2, 4-dinitrophenylhydrazine.

Statistics

The software Graphpad Prism V for Windows (Graphpad Software, San Diego, CA, USA) was used for statistical calculations. The MDA and NOx concentrations in the plasma had skewed distributions to high values, mainly in the irradiated groups. Therefore, the results are given as the geometric mean (range). One-way analysis of variance (ANOVA) and the Dunnett's multiple comparison tests were used for between-group comparisons of the logarithmically transformed MDA and NOx concentrations. The plot of the Kaplan–Meier estimate of the survival function was constructed. The log-rank test was used to test for the difference between survival functions of the control group (I-PBS) and each of the protected groups (I-L-NAME, I-ALC, I-AG). The significance level was set at α=0.05.

Results

Survival study

For the group irradiated with 8 Gy and pretreated with PBS (I-PBS), the survival proportion was 34.6 % (9/26) by day 30. In the I-L-NAME, I-ALC and I-AG groups, the survival proportions were 75 % (15/20), 75 % (15/20) and 55.6 % (5/9), respectively. The corresponding improvement in survival relative to the unprotected I-PBS group was 2.6-fold (I-L-NAME and I-ALC) and 1.6-fold (I-AG). The Kaplan-Meier curves are shown in Figure 1. According to the log-rank test, L-NAME and ALC significantly improved survival if compared to the I-PBS group ($P<0.005$ for both), and their survival curves were almost superimposable. The difference in the survival rate between the I-AG and I-PBS

groups did not reach significance (P=0.17). The median survival time achieved 14 days in the I-PBS rats, whereas it exceeded the 30-day observation period in the I-L-NAME, I-ALC and I-AG groups. The mean (SD) survival times of animals killed by irradiation in the respective groups were 12.4 (2.9), 14.4 (1.9), 15.2 (2.4), and 15.5 (3.1) days (P=0.13).

The effect of radioprotective agents on plasma NOx

In the PBS controls (C-PBS), the geometric mean (range) plasma NOx concentration achieved 9.6 µmol/l (5.3-12.4 µmol/l). No significant change of plasma NOx was observed in the groups of non-irradiated rats injected with ALC and AG (Figure 2). Its reduction by 28 % caused by L-NAME was marginally significant (P=0.085). Whole body irradiation of the I-PBS group increased the plasma NOx 1.9-fold above that of the C-PBS group (geometric mean: 18.4 µmol/L, range: 9.0-52.4 µmol/L, P<0.05). Of the radioprotective agents, only L-NAME prevented the increase of plasma NOx. The NOx concentration (geometric mean: 8.6 µmol/l, range: 7.3-10.8 µmol/l) of the I-L-NAME group was comparable to that of C-PBS rats, and it was significantly less than in I-PBS rats (P<0.05 vs I-PBS). The I-AG and I-ALC groups did not differ from the I-PBS group. Compared to the C-PBS group, the NOx concentration of I-ALC rats (geometric mean: 24.9 µmol/l, range: 12.9-38.7 µmol/l) was increased 2.6-fold (P<0.01 vs. C-PBS) and that of the I-AG group 1.7-fold (geometric mean: 16.7µmol/l, range: 11.9-29.2 µmol/l, P<0.05 vs. C-PBS) (Fig.2).

The effect of radioprotective agents on plasma MDA

A single dose injection of either L-NAME or AG to non-irradiated rats (the groups C-L-NAME and C-AG) increased the concentration of plasma MDA 1.7-fold above that of the C-PBS group (P<0.001 vs C-PBS for both NOS inhibitors) while pretreatment with ALC caused no change (Figure 3). The geometric mean (range) plasma MDA concentration (µmol/l) was 2.5 (1.8-4.6), 4.4 (3.2-5.1), 4.3 (3.8-5.2), and 2.3 (1.8-2.6), in C-PBS, C-L-NAME, C-AG, and C-ALC groups, respectively. Whole body irradiation resulted in an increased MDA concentration 1.8-fold in the I-PBS group as compared to C-PBS rats (P<0.001). The only agent which completely prevented the radiation-induced increase of plasma MDA was ALC (the I-ALC group geometric mean: 1.8 µmol/l, range: 1.6-2.1, P<0.001 vs I-PBS). Pretreatment with L-NAME exerted no effect. A small MDA-reducing effect of AG could be observed. However, the difference between the I-AG and I-PBS group did not reach significance (Fig.

3). The geometric mean (range) plasma MDA concentration (μmol/l) of I-L-NAME and I-AG groups remained elevated above that of the C-PBS group [4.2, (3.7-5.0), P<0.001 and 3.5, (3.1-4.7), P<0.05].

Discussion

In this study, single i.p. injection of Wistar rats with 100 mg/kg L-NAME before the whole body irradiation with 8 Gy resulted in a 2.6-fold reduced risk for death during the subsequent 30-day period. The NOS inhibitor effectively prevented the radiation-induced elevation of NO production as documented by plasma NOx at 6 h following exposure. On the contrary, the concentration of plasma MDA of irradiated rats injected with L-NAME and PBS was similar indicating no effect of L-NAME on the extent of lipoperoxidation augmented by irradiation. Injection of rats with 400 mg/kg AG, the second NOS inhibitor under study, caused only a modest decrease in MDA level and failed to reduce plasma NOx. The chance for survival improved 1.6-fold after AG, but this difference was only marginally significant. Pretreatment of rats with ALC for 5 days preceding irradiation completely prevented the increase in plasma MDA and caused an improvement in the survival rate similar to that observed after L-NAME.

L-NAME is a methyl ester prodrug that is rapidly bioactivated to N(omega)-nitro-L-arginine. In vitro, N(omega)-nitro-L-arginine exhibits a greater inhibitory potency towards nNOS and eNOS than iNOS, whereas AG is partially iNOS-selective (Alderton et al. 2001). After the relatively high doses of both compounds, however, it would be unrealistic to expect that a single NOS isoform was selectively inhibited without reducing the activity of others. Radioprotective effects of both NOS inhibitors were studied at the doses selected with the help of the supportive data published by others. Mice injected with 100 mg i.p N(omega)-nitro-L-arginine 15-60 min before exposure were partially protected from the lethal effect of 10 Gy whole body irradiation with survival proportions between 25 and 70 % (Liebmann et al. 1994). Two injections of AG (100 mg/kg) at 10 and 60 min before irradiation of mice with 23 Gy prolonged the median survival time from 7.6 to 12.4 days without increasing the 30-day survival from 0 % (Ohta et al. 2007). AG at a dose of 50-800 mg/kg administered by the gavage route 2 h before whole-abdominal irradiation of rats with 12 Gy reduced diarrhea and small-bowel damage with the maximum radioprotective effect at 400 mg/kg (Huang et al. 2009).

Despite a high dose used for radioprotection, single i.p. injection of AG was ineffective or markedly less effective than L-NAME in preventing the radiation-induced increase in plasma NOx and mortality. The acute toxicity of AG is low. In one study, the LD50 of single i.p. administration of AG was

803 mg, and once daily i.p. injection of 321 mg for 7 days did not affect survival, behaviour and body weight of rats (Sugiyama et al. 1986). The extent of the increase in plasma NOx at 6 h after 8 Gy agrees with the radiation dose – concentration relationship described in our previous work (Babicova et al. 2011). The lower NOx-reducing and radioprotective effects of AG can be explained, most likely, by its lower inhibitory activity towards all NOS enzymes and the constitutive forms eNOS and nNOS in particular. Our unpublished data document that radiation-induced elevation of plasma NOx is less attenuated by a highly iNOS-selective and potent inhibitor 1400W (geometric mean plasma NOx 11.2 μmol/l) than L-NAME (8.6 μmol/l) despite the high dose of 10 mg/kg which was shown by others to effectively decrease plasma NOx in endotoxemic rats (Wray et al. 1998). It can be deduced that both constitutive NOS and iNOS contribute significantly to the levels of plasma NOx after whole body irradiation.

Besides selectivity, the differences in potency as well as pharmacokinetics between AG and L-NAME might have influenced the study outcome. After an i.v. bolus dose to rats, the half-life of N(omega)-nitro-L-arginine is approximately 20 hours. Following a single bolus injection of its prodrug L-NAME, an effective NOS inhibition likely persisted over 72 h or longer (Piotrovskij et al. 1993). This contrasts with a rapid kinetics of AG elimination. High concentrations of AG were detected in organs and tissues at 5 min after an i.v. injection to rats, and their decline followed the first-order kinetics with the half-life of 90 min (Beaven et al. 1969). No alteration in the plasma concentration of NOx was detected in the C-AG group, i.e. after injection of AG to nonirradiated rats. The small NOx-reducing effect of L-NAME in nonirradiated animals was only marginally significant. Other investigators reported similar findings. AG or L-NAME given at a dose of 100 mg/kg at 0, 3, 6, 10, and 20 h following cecal ligation and puncture attenuated the increase of plasma NOx in septic rats without any measurable effect in intact animals (Eum et al. 2006).

In addition to NOS inhibition, AG can be beneficial due to its antioxidant and free radical scavenging characteristics, inhibition of diamine oxidase and prevention of oxidant-induced apoptosis (Nilsson 1999; Chowdhury et al. 2009; Polat et al. 2006; Fan et al. 2010). The α,β-dicarbonyl scavenging activity of AG dampers the formation of the highly reactive advanced glycosylation end products from proteins. Due to this characteristic, AG was evaluated in a clinical trial with the aim to prevent complications of diabetes mellitus (Bolton et al. 2004). In this study, AG caused only a small decrease of the plasma MDA in irradiated rats. Of note, administration of both NOS inhibitors had pro-

oxidant effects in nonirradiated groups. Numerous studies brought the evidence of an increased oxidative stress in response to NOS inhibitors that occurs on the cellular level as well as in vivo. The oxidative stress is triggered by several mechanisms including a drop in scavenging of reactive oxygen species at decreased levels of NO (Wink et al. 2001), direct reduction by NOS inhibitors of cellular antioxidant capacity (Riganti et al. 2003), and an increased leukocyte–endothelial cell adhesion and microvascular permeability (Kurose et al. 1995).

In the present study, only pretreatment with ALC significantly attenuated the radiation-induced elevation of oxidative stress as indicated by plasma MDA at 6 h following exposure. Similar modulatory effect on the MDA levels in both the lung and liver at 24 h folowing whole body irradiation of rats was achieved by administration of a daily dose of 250 mg/kg ALC for 5 consecutive days in a study by Mansour (Mansour 2006). Moreover, the investigator found a significant increase in the tissue activities of antioxidant enzymes (superoxide dismutase and glutathione peroxidase), and of the concetration of reduced glutathione in response to ALC pretreatment of rats before irradiation. In addition, ALC completely abolished the radiation-induced increase of tissue NOx levels (Mansour 2006). This contrasts with our results showing that plasma NOx at 6 h post-irradiation further increased when irradiated rats were given ALC. We have no direct data to explain this contradictory observation. However, in rats with a physiological or reduced concentration of plasma NOx (Wistar-Kyoto and spontaneously hypertensive rats), long term supplementation with L-carnitine increases plasma antioxidant capacity and plasma NOx (Gómez-Amores et al. 2007). In patients with peripheral arterial disease, decreased levels of plasma NOx augment after repeated administration of propionyl-L-carnitine, and the plasma concentrations of the oxidative stress biomarker 8-hydroxy-2-deoxy-2-deoxyguanosine and plasma NOx are inversely correlated (Loffredo et al. 2007). Plasma NOx of healthy subjects is increased following oral treatment with glycine propionyl-L-carnitine (Bloomer et al. 2009). The physiological mechanisms leading to an increased systemic availability of NO described in the above studies after administration of carnitine and its esters remain to be elucidated. The reduced ROS formation may result in less consumption of NO or its elevated formation from eNOS, due to a decreased oxidative uncoupling of bioactive dimeric eNOS to its inactive monomeric form (Xia et al. 1998).

Conclusion

The results indicate that pretretment with both the nonspecific NOS inhibitor L-NAME and the antioxidant ALC provide significant protection of rats against whole body irradiation. The partially iNOS-selective inhibitor and antioxidant AG given in a single dose before exposure was significantly less effective in increasing the 30-day survival, presumably due to its short half-life. The reduced risk for death may be directly related to effective attenuation of the radiation-induced elevation of oxidative stress by ALC and of NO production by L-NAME during early response to ionizing radiation as indicated on the systemic level by MDA and NOx plasma concentrations. The lack of correlation between the NOx and MDA results is concordant with the view that ROS function as initiators and NO and other RNS as effectors in multiple signal transduction pathways (Mikkelsen and Wardman 2003), and that significant radioprotection can be afforded by suppression of the former or latter.

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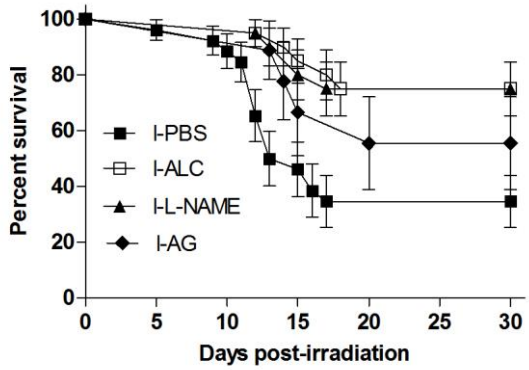

Fig. 1: Kaplan–Meier survival curves showing a 30-day survival of whole-body irradiated (8 Gy) rats injected with phosphate-buffered saline (I-PBS, n=26), 100 mg/kg L-NAME (I-L-NAME, n=20), 400 mg/kg aminoguanidine I-AG, n=9) and 250 mg/kg acetyl-L-carnitine (I-ALC, n=20). PBS, L-NAME and AG were administered i.p. 1 h before irradiation. ALC was injected i.p. once daily over 5 days with the last dose 1 h before P<0.005, I-ALC vs I-PBS; P=0.17, I-AG vs I-PBS.

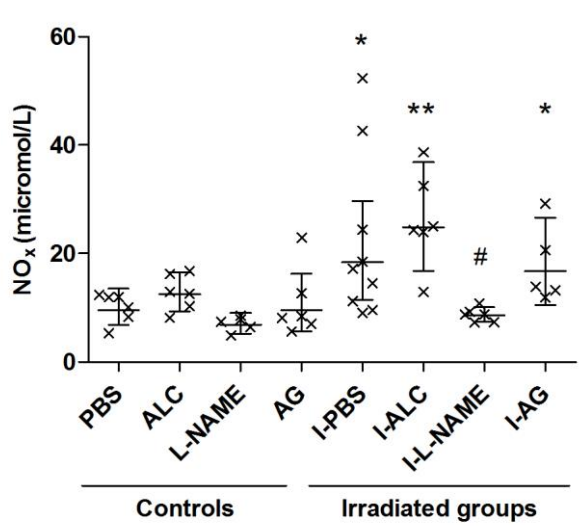

Fig. 2: The sum of nitrite and nitrate concentrations (NOx) in the plasma of irradiated and control rats 6 h after whole-body gamma irradiation delivered to the total dose of 8 Gy. Horizontal lines represent geometric mean concetrations and error bars indicate the 95-% confidence interval for the mean (N = 5-9 rats per group). Results of the Dunnett's multiple-comparison test: *…P<0.05, **…P<0.01 from comparison of all groups with the control PBS group, #...P<0.05 from comparison of irradiated groups I-ALC, I-L-NAME and I-AG, respectively, with the irradiated PBS group. Symbols: NOx…total concentration of nitrite+nitrate, PBS…phosphate-buffered saline, L-NAME…N(omega)-Nitro-L-

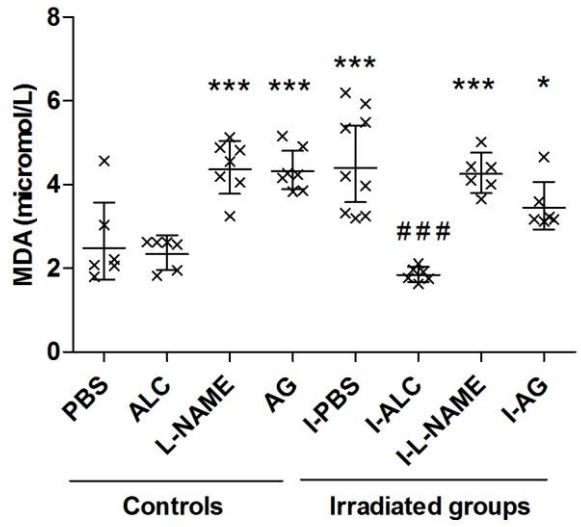

Fig. 3: Malondialdehyde concentration (MDA) in the plasma of irradiated and control rats 6 h after whole-body gamma irradiation delivered to the total dose of 8 Gy. Horizontal lines represent geometric mean concentrations and error bars indicate the 95-% confidence interval for the mean (N = 5-9 rats per group). Results of the Dunnett's multiple-comparison test: *…P<0.05, ***…P<0.001 from comparison of all groups with the control PBS group; ###...P<0.001 from comparison of irradiated groups I-ALC, I-L-NAME and I-AG, respectively, with the irradiated PBS group. Symbols: MDA…malondialdehyde, PBS…phosphate-buffered saline, L-NAME…N(omega)-Nitro-L-arginine methyl ester;