ORIGINAL ARTICLE

EFFECT OF ACETYL-L-CARNITINE PRETREATMENT ON RADIATION PNEUMONITIS AND L-ARGININE-NO METABOLIC PATHWAY

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Summary

Purpose: The purpose of our study is to examine the effect of acetyl-L-carnitine (ALC) pretreatment on radiation pneumonitis and L-arginine-nitric oxide (NO) metabolic pathway after local chest irradiation.
Materials and methods: Female Wistar rats were pretreated with either phosphate buffer saline or ALC and locally irradiated by a dose of 15 Gy onto the chest. Samples were collected 7 weeks after irradiation. Exhaled NO was monitored using chemiluminiscence method. In plasma and bronchoalveolar lavage (BAL) fluid, nitrite/nitrate (NO_x) and malondialdehyde were measured by high-performance liquid chromatography. Total protein concentration was assayed colorimetrically. In lungs, inducible and endothelial nitric oxide synthase (iNOS, eNOS), arginase (Arg) I and II, cationic amino acid transporter (CAT) 1, 2 and 3 relative mRNA expression were measured by RT-PCR. Expression of Arg I and II was also evaluated by Western blot. Histochemical analysis was used to measure air/tissue ratio and neutrophil infiltration.
Results: ALC pretreatment increased survival rate of irradiated rats. It also decreased NO_x concentration in BAL, while in lungs, we observed increased air/tissue ratio, reduced neutrophil infiltration, decreased CAT 1 and CAT 3 mRNA expression, and reduced Arg I and Arg II expression.
Conclusions: ALC pretreatment improves clinical prognosis and seems to reduce intensity of post-radiation changes on a molecular and microscopic level.

Key words: ionising radiation; acetyl-L-carnitine; NO, NO synthase; arginase; cationic amino acid transporter; lung

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ABBREVIATIONS

ALC – acetyl-L-carnitine; Arg – arginase; BAL – bronchoalveolar lavage; CAT – cationic amino acid transporter; eNOS – endothelial nitric oxide synthase; iNOS – inducible nitric oxide synthase; NO – nitric oxide; RP – radiation pneumonitis; SPF - specific pathogen free

INTRODUCTION

Radiation pneumonitis (RP) is one of the major dose-limiting complications during radiotherapy to the thoracic region. RP is an acute inflammatory reaction that is characterized by alveolar cell depletion, interstitial oedema and inflammatory cell accumulation and develops within weeks after irradiation. It occurs in 5-15% of patients treated with thoracic radiotherapy, especially in children or when a more aggressive approach such as combined chemoradiotherapy is used [1 - 4].

Measurement of exhaled nitric oxide (NO) is a noninvasive method used to predict onset of radiation pneumonitis in irradiated patients and generally, a method used to assess inflammatory status and response to therapy in many acute and chronic lung diseases [5 - 8]. NO is a small gas molecule with many various biological functions [9]. The synthesis of NO is carried out from L-arginine by nitric oxide synthases. In lungs, NO is constitutively produced by endothelial nitric oxide synthase (eNOS), but when exposed to proinflammatory cytokines or lipopolysaccharide, the NO production rises via inducible nitric oxide synthase (iNOS) in epithelial and inflammatory cells [10 - 12]. One of the factors that might affect NO synthesis is L-arginine cellular uptake, which is regulated by cationic amino acid transporter (CAT) [13]. So far, three members of the CAT family participating in L-arginine cellular uptake have been identified, CAT 1, 2 and 3 [14]. CAT 1 is constitutively expressed, while CAT 2 is the inducible transporter and its expression is upregulated in macrophages upon immune activation [15, 16]. The importance of CAT 3 remains unknown [14, 17]. Other factors that may limit NO synthesis are arginases. There are two isoforms of arginases in mammalian cells: arginase I (Arg I) is a cytosolic enzyme abundant in liver, and arginase II (Arg II) is a mitochondrial enzyme with widespread tissue distribution [18]. Arginases compete for L-arginine substrate by metabolizing it to L-ornithine within the urea cycle and this competition seems to help regulate the inflammatory process [19 - 22].

The aim of our work is to evaluate the effect of acetyl-L-carnitine (ALC, 3-hydroxy-4-trimethylammoniumbutyric acid) pretreatment on radiation pneumonitis and L-arginine-NO metabolic pathway 7 weeks after local chest irradiation. Exhaled NO is evaluated in the contxt of nitrite/nitrate, malondialdehyde and total protein concentration measurements in plasma and bronchoalveolar lavage fluid (BAL) and expression of genes and proteins participating in NO production in lungs.

MATERIAL AND METHODS

Animals

Specific pathogen free 12-16 month old female Wistar rats weighing 165-272g (Velaz Ltd, Prague, Czech Republic) were used during the experiments. Animals were fed with standard feed ST-1 (Velaz Ltd, Prague, Czech Republic) and water ad *libitum*. The rats were kept in an air-controlled room (22 ± 2°C and 50 ± 10% relative humidity) with a 12hour light/dark cycle with light from 7:00 to 19:00 h. All procedures were approved by the Ethical Committee of the Faculty of Military Health Sciences Hradec Kralove (Czech Republic).

Pretreatment

The animals were divided into 2 groups of 10 animals: a control group administered with phosphate buffer saline (PBS, Sigma Aldrich, Prague, Czech Republic), and a group administered with acetyl-L-carnitine (ALC, Sigma Aldrich). ALC was dissolved in PBS and intraperitoneally (i.p.) administered 5 days before irradiation at 5 single doses of 250 mg/kg (5 x 250 mg/kg). The last dose was applied 1 hour before irradiation.

Irradiation

The animals were irradiated using a ^{60}Co unit (Chirana, Prague, Czech Republic) at a dose rate of 2.61 Gy.min^{-1} with a target distance of 0.5 m. Dosimetry was performed using an ionization chamber (Dosemeter PTW Unidos 1001, Serial No. 11057, with ionization chamber PTW TM 313, Serial No. 0012; RPD Inc., Albertville, MN, USA). Before irradiation, the rats were analgosedated with ketamine (5%, 40 mg/kg) and xylazine (2%, 4.8 mg/kg) (both BIOVETA, Ivanovice na Hane, Czech Republic). Under analgosedation, the animals were fastened onto a Plexiglas underlay (VLA JEP, Hradec Kralove, Czech Republic) to assure consistent unilateral back-front exposure onto the chest with the rest of the body shielded with lead. The chest was irradiated with a single dose of 15 Gy (1 animal was irradiated each time).

Sample collection

At the end, 7 weeks after irradiation, animals were i.p. anaesthetized with sodium pentobarbital sodium salt at a dose of 50 mg/kg (Sigma Aldrich). Blood was collected into the 9-mL S-monovette

K3EDTA (Sarstedt Ltd, Prague, Czech Republic) from abdominal aorta. The whole blood was centrifuged (2000 g force, 10 min, 4°C; Rotina 46R Hettich zentrifugen, Hettich Laborapparate AG, Bäch, Germany) and plasma was stored in -80°C for further analysis. After exsanguination, bronchoalveolar lavage fluid (BAL) was obtained by instilling 10 ml of 0.9% saline solution (Sigma Aldrich) through the tracheostomy. BAL was centrifuged (2000 g force, 10 min, 4°C; Rotina 46R Hettich zentrifugen) and supernatant was stored in -80°C for further analysis. Finally, lungs were removed; left lungs were immediately frozen in liquid nitrogen (Linde gas Ltd., Prague, Czech Republic) and stored in -80°C for western blot analysis and RT-PCR. Right lungs were carefully fixed with 10% neutral buffered formalin (Chemapol, Prague, Czech Republic) for histological analysis.

Measurement of exhaled nitric oxide (eNO)

Before the final sample collection, exhaled nitric oxide (eNO) was measured using a chemiluminescence analyzer (CLD 88, EcoMedics, Deutern, Switzerland) with a sensitivity of 0.1–5000 ppb. The method is a procedure described for mice by Ahmad et al. with minor modifications [23]. Briefly, rats were individually put into a sealed plastic box with a volume of 3 L (P-Lab, Prague, Czech Republic). The box was ventilated for approximately 3 min with NO-free air from the Denox module of the analyzer. Thereafter, air from the box was sampled to confirm zero baseline. The box was tightly closed and after 10 min, air was sucked from the box into the analyzer at a rate of 330 ml/min being replaced by NO-free air from the Denox and an initial stable concentration of eNO was recorded.

Measurements of nitrate/nitrite concentration in plasma and BAL

The sum nitrate and nitrite concentration (NO_x), an indicator of NO concentration, was measured by high-performance liquid chromatography (HPLC) with fluorescence detection (Agilent, Santa Clara, CA, USA) as described by Woitzik et al. [24]. The method is based on enzymatic reduction of nitrates by nitrate reductase (from *Aspergillus niger*; Sigma Aldrich) and on the determination of nitrites after derivatization with 2,3-diaminonaphtalene (Sigma Aldrich).

Measurements of malondialdehyde (MDA) concentration in plasma and BAL

The concentration of malondialdehyde, an indicator of oxidative stress in organism, was measured using a non-enzymatic method by Pilz et al. [25]. In brief, plasma or BAL samples were mixed with 6 M sodium hydroxide (Sigma Aldrich) for alkaline hydrolysis (60°C, 30min). Subsequently, perchloric acid (Sigma Aldrich) was added for deproteinization. After centrifugation (15000 g force, 10 min, 4°C, thermo scientific IEC CL31R Multispeed Centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, UA), the supernatant was mixed with 2,4 dinitrophenylhydrazine (Sigma Aldrich) to form hydrazine, which was analyzed using HPLC system with ultraviolet detection (Agilent).

Measurements of total protein concentration in plasma and BAL

Total protein concentration in plasma and BAL was measured colorimetrically using BCA Protein Assay Kit (Pierce, Rockford, IL, USA) in 96-well microplate (40 μL sample/180 μL BCA working reagent, 37°C/30 min incubation, and 570 nm). The standards were prepared from a stock solution of BSA (Sigma Aldrich).

Measurement of iNOS, eNOS, Arg I, Arg II, CAT 1, CAT 2, and CAT 3 expression using RT-PCR method

Frozen lung tissue was homogenized using the homogenizer MagNA Lyser (Roche) in RLT buffer (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The total RNA was isolated from lung tissue using the RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions. Concentration and purity of isolated RNA was measured by spectrophotometer NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). RNA was reversely transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, California, USA). The reaction was carried out at 25°C for 10 min, at 32°C for 120 min, at 85°C for 5 s and terminated at 4°C (2720 Thermal Cycler; Life Technologies Corporation). For the quantitative analysis, we used 7500 Fast Real-Time PCR System (Life Technologies Corporation). Primers and probes for target genes (Table I) labeled with the reporter dye molecule FAM were synthesized by Applied Biosystems (Life Technologies Corporation).

Table I: Target genes.

target genes	gene symbol	gene name	assay ID
iNOS	Nos2	nitric oxide synthase 2, inducible	Rn00561646_m1
eNOS	Nos3	nitric oxide synthase 3, endothelial cell	Rn02132634_s1
ARG I	ArgI	arginase, liver	Rn00567522_m1
ARG II	ArgII	arginase type II	Rn00569814_m1
CAT 1	Slc7a1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	Rn00565399_m1
CAT 2	Slc7a2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	Rn01488325_m1
CAT 3	Slc7a3	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3	Rn00500256_m1
GAPDH	Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Rn01775763_g1

Every PCR contained 2X TaqMan Universal PCR Master Mix, 20X TaqMan Assay Mix (Life Technologies Corporation), and 30 ng of cDNA template and was conducted according to this amplification protocol: 1 cycle for initial denaturation (3 min, 95°C), followed by 50 amplification cycles of denaturation (10 s, 95°C), annealing and extension (10 s, 60°C). Housekeeping RT-PCR was performed to determine the expression of glyceraldehyde-3-phosphate dehydrogenase (Life Technologies Corporation). The relative expression ratio was then calculated from the ΔCt_{target} and $\Delta Ct_{housekeeping}$ values as described previously [26](Fuksa et al. 2010).

Western Blot analysis of Arg I and Arg II expression in lung tissue

Frozen lungs were homogenized with homogenizer Ultra Turrax (IKA Werke GmbH & Co. KG, Staufen, Germany) in 5 volume of cold homogenization buffer [50 mM TRIS, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 1.5 µM Aprotitin, 14.5 µM Pepstatin A (all Sigma Aldrich) and 23.4 µM Leupeptin (Serva, Heidelberg, Germany)]. Supernatant was collected after centrifugation (1000 g force, 10 min, 4°C; Rotina 46R Hettich zentrifugen) and ultracentrifugation (19000 g force, 30 min, 4°C; Sorvall centrifuges Discovery M120Se Hitachi). Total protein concentration in the supernatant was determined using BCA protein assay kit (Pierce). Pooled samples were prepared from tissue homogenates of all animals in the respective groups using the volumes of the supernatants containing the same amount of total protein. Proteins were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis using 30 µg of protein per sample. Resultant proteins were electroblotted onto polyvinylidenedifluoride membranes (Bio-Rad Laboratories, Prague, Czech Republic). After blotting, membranes were incubated for 1 h at room temperature in blocking solution [5% nonfat dry milk (Bio-Rad Laboratories) added to 0.05% Tween 20 in tris-(hydroxymethyl)aminomethane-buffered saline (TBS/T, both Sigma Aldrich)]. After blocking, the membranes were washed three times in 0.05% TBS/T and incubated overnight (4°C) with primary antibodies: mouse monoclonal anti-Arg I antibody (1:1000, BD Transduction Laboratories, San Jose, CA, USA), rabbit polyclonal anti-Arg II antibody (1:500, H-64, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse monoclonal anti-ß-actin antibody (1:20000, Sigma Aldrich). Subsequently, the membranes were washed three times in 0.05% TBS/T and incubated with HRP-conjugated secondary antibodies at room temperature for 1 h: goat polyclonal anti-mouse antibody (1:25000; Dako, Glostrup, Denmark) was used for Arg I and ß-actin primary antibodies and swine polyclonal anti-rabbit antibody (1:2000; Dako, Denmark A/S, Denmark) was used for Arg II primary antibody. The blots were washed three times with TBS buffer and developed with enhanced electrochemiluminiscence (Roche, Prague, Czech Republic).

Histological examination of lung tissue

After formalin fixation, lung samples were embedded into paraffin (Paramix, Holice, Czech Republic). Tissue sections 5 µm thick were cut (Microtome model SM2000 R, Leica, Heidelberg, Germany) and stained with haematoxylin-eosin (both Merck, Prague, Czech Republic) for the air/tissue ratio measurement and with Naphthol AS-D Chloroacetate (Specific Esterase) Kit (Sigma Aldrich) to detect neutrophil granulocytes. The air/tissue ratio and neutrophil granulocytes were measured in 15 randomly selected viewing

fields per sample at 400-fold magnification using a BX-51 microscope (Olympus, Prague, Czech Republic) and ImagePro 5.1 computer image analysis system (Media Cybernetics, Bethesda, MD, USA).

Statistical analysis

The Mann-Whitney (SigmaStat 3.1, Systat Software Inc., Erkhart, Germany) test was used for the statistical analysis giving mean $\pm 2 \times$ SE (standard error of mean). Mortality rates were e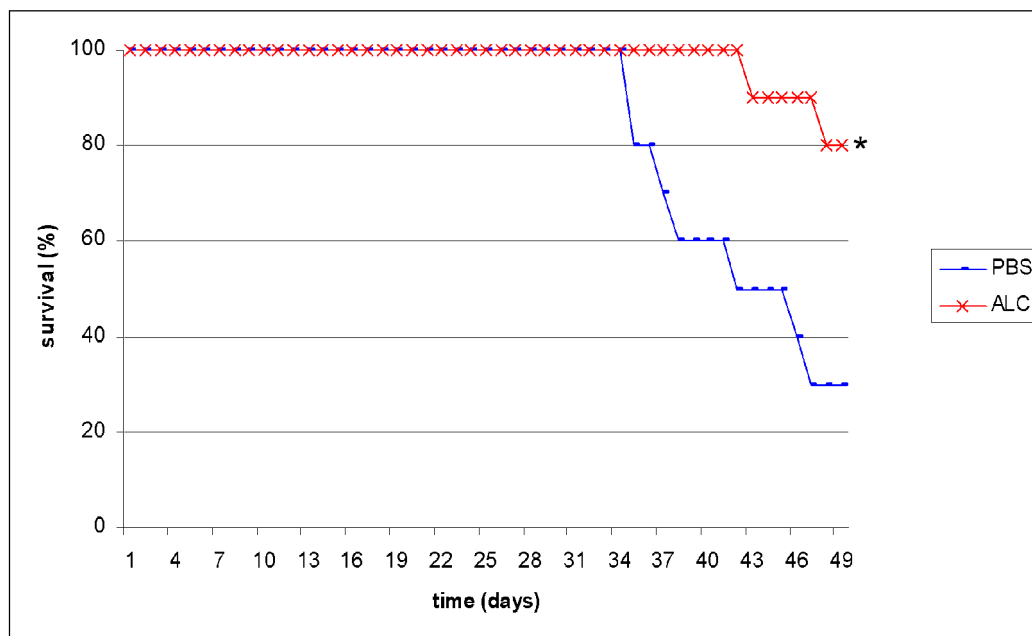stimated by the Kaplan-Meier method and compared by a log-rank test (SigmaStat 3.1). The differences were considered significant when $p \leq 0.05$.

RESULTS

Survival

Our results show significantly increased survival rate in ALC treated group (80%, $p \leq 0.05$) when compared with PBS pretreated animals (30%). (Figure 1)

Figure 1. Animal survival

Significant differences between control and ALC groups: * $p \leq 0.05$.

Effect of ALC pretreatment on NO concentration in exhaled air (eNO)

In comparison with control animals, we did not observe any significant effect of ALC pretreatment on eNO concentration 7 weeks after local chest irradiation. (Table 1)

Table 1. Average values of eNO concentration $\pm 2 \times$ SE.

	PBS (control)	ALC
eNO, parts per billion	0.64 ± 0.26	0.64 ± 0.12

Effect of ALC pretreatment on nitrate/nitrite, malondialdehyde and total protein concentration in plasma and BAL

When compared with control values, we measured significantly decreased nitrate/nitrite concentration in BAL of ALC pretreated rats. Nitrate/nitrite concentration decreased by 22% ($p \leq 0.05$). On the other hand, ALC pretreatment did not show any significant effect on nitrate/nitrite concentration in plasma and malondialdehyde and total protein concentration in both plasma and BAL 7 weeks after local chest irradiation by a dose of 15 Gy. (Table 2)

Table 2. Average values of nitrate/nitrite (NO_x), malondialdehyde (MDA) and total protein concentrations (TP) in plasma and BAL $\pm$ 2 $\times$ SE.

	PBS (control)	ALC
Plasma		
NOx, µmol/L	21.2 ± 27.4	17.7 ± 11.1
MDA, µmol/L	1.84 ± 0.20	2.03 ± 0.22
TP, g/L	64.0 ± 0.92	59.2 ± 3.30
BAL		
NOx, µmol/L	6.29 ± 0.24	4.88 ± 0.21 *
MDA, nmol/L	236 ± 85	408 ± 151
TP, mg/L	490 ± 550	1090 ± 520

Significant differences between control and ALC groups: * $p \leq 0.05$.

Effect of ALC pretreatment on iNOS, eNOS, Arg I, Arg II, CAT 1, CAT 2, and CAT 3 mRNA expressions in lungs

Our results indicate significantly decreased CAT 1 and CAT 3 mRNA expression in pretreated animals with values being decreased by 41% ($p \leq 0.05$) and 47% ($p \leq 0.05$), respectively. Expressions of iNOS, eNOS, Arg I, Arg II, and CAT 2 in ALC pretreated group were not significantly changed. (Table 3)

Table 3. Average values of iNOS, eNOS, arginase I and II and cationic amino acid transporter (CAT) 1, 2 and 3 relative mRNA expression in lungs $\pm$ 2 $\times$ SE.

	Relative mRNA expression, %	
	PBS (control)	ALC
iNOS	100 ± 80	138 ± 111
eNOS	100 ± 26	99 ± 46
Arginase I	100 ± 105	57 ± 16
Arginase II	100 ± 22	86 ± 51
CAT 1	100 ± 31	59 ± 13 *
CAT 2	100 ± 23	64 ± 27
CAT 3	100 ± 20	53 ± 23 *

Significant differences between control and ALC groups: * $p \leq 0.05$.

Effect of ALC on Arg I and Arg II protein expression in lungs

In the Western blotting experiments, we detected decreased both Arg I and Arg II protein expression in ALC pretreated group. (Figure 2)
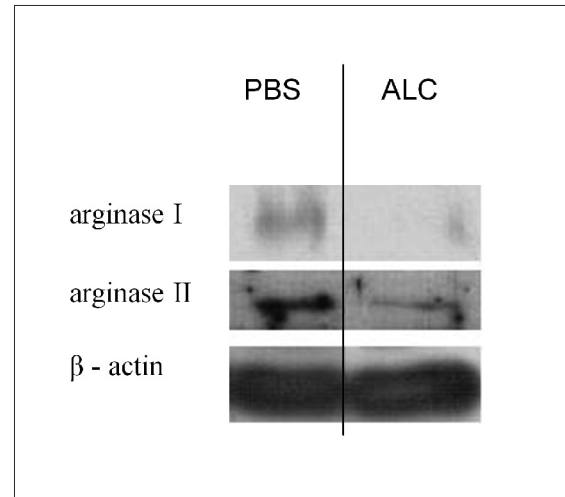
Figure 2. Results of western blot analysis of arginase I and II expression in lung tissue.

Effect of ALC on neutrophil infiltration and air/tissue ratio

When compared with PBS treated animals, we observed a significantly higher air/tissue ratio and a reduced number of neutrophil granulocytes in rat lungs in ALC pretreated group. (Table 4)

Table 4. Average values of neutrophil infiltration and air/tissue ratio in lungs $\pm$ 2 $\times$ SE per microscopic field.

	PBS (control)	ALC
Air/tissue ratio, %	63 ± 2	67 ± 2 *
Number of neutrophils	21 ± 3	12 ± 1 *

Significant differences between control and ALC groups: * $p \leq 0.001$.

DISCUSSION

ALC is a small water-soluble molecule, which facilitates the transfer of long-chain fatty acids into the mitochondria and is crucial for mitochondrial energy production [27, 28]. It also stimulates acetylcholine production, protein and phospholipid

synthesis, and has been shown to have an anti-oxidative effect [27]. ALC protects cells from oxidative damage by a few different mechanisms, including stabilization of mitochondrial function, stabilization of superoxide dismutase activity and damaged cell membranes, reduction of reactive oxygen species formation produced by the xanthine/xanthine oxidase system, and

enhancement of capacity of non-enzymatic antioxidants [29 - 32]. In lungs, ALC pretreatment decrease oxidative stress early after irradiation [33], which, according to our results, seems to improve clinical prognosis 7 weeks after irradiation and seems to reduce the intensity of post-radiation changes during an initial phase of radiation pneumonitis on a microscopic level (Fig 3, 4).
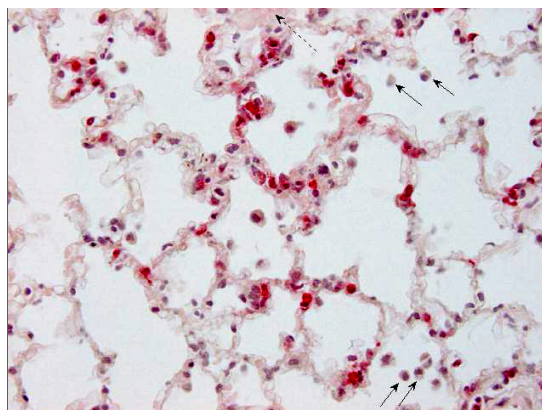

Figure 3. Sample of rat lungs irradiated by 15 Gy and collected 7 weeks after irradiation. Red colour (chloroacetate esterase positivity) indicates neutrophyle granulocytes. In the microphotograph, unspecific signs of radiation damage can be observed, including neutrophil granulocyte infiltration, pneumocyte desquamation (solid arrow), and fluid exudation (dashed arrow).
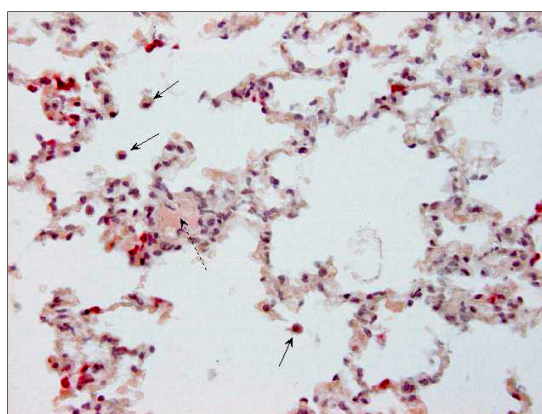

Figure 4. Sample of rat lungs pretreated with acetyl-L-carnitine, irradiated by 15 Gy, and collected 7 weeks after irradiation. Unspecific signs of radiation damage [neutrophil granulocyte infiltration, pneumocyte desquamation (solid arrow), and fluid exudation (dashed arrow)] can be still observed, nevertheless, the amount of neutrophil granulocytes decreased.

In humans, radiation pneumonitis has been associated with increased exhalation of NO [7]. Moreover, the elevation of eNO on the last day of radiotherapy predicts subsequent symptomatic radiation pneumonitis weeks to months after treatment [7]. The SPF Wistar rat model appears to

be different. Zhang et al. did not find any significant change of iNOS expression in SPF Wistar rats 2 - 24 weeks after local chest irradiation by 12 Gy [34]. This correlates with our unpublished data showing no significant change of eNO concentration 1 - 7 weeks after local chest irradiation by 15 Gy. It seems

that local high-dose irradiation onto the chest does not affect iNOS expression and eNO concentration in SPF Wistar rats during the initial phase of radiation pneumonitis. It may, therefore, be the reason why we did not measure any effect of ALC pretreatment on both parameters. Interestingly, we found decreased nitrate/nitrite concentration in BAL of ALC pretreated animals. Since the expression of both nitric oxide synthases and NO production were not changed, the mechanisms/reason reducing nitrate/nitrite concentration in BAL of ALC pretreated rats remains uncertain. Possibly, it could be related to the different intensity of acute inflammatory reaction observed between the two groups – a higher air/tissue ratio in ALC treated group might indicate a lower volume of fluid exudation into lung alveoli and consequently, a lower amount of nitrates and nitrites collected into BAL.

In comparison to uninfluenced NO production in SPF rats, ionizing radiation affects other molecules participating in arginine-NO metabolism. Zhang et al. measured increased Arg I protein expression 1 – 24 weeks after irradiation [34]. And although we have not found any relevant information on the relation between ionizing radiation and Arg II, CAT 1, 2, or 3 expression in SPF rats, for instance, Arg II is strongly induced during experimental asthma and ovalbumin-induced airway inflammation in mice [35, 36]. Moreover, according to our unpublished data, local chest irradiation by a dose of 20 Gy increases CAT 1 and 3 expressions 2.1- and 7.4-fold, respectively. This indicates that increased both arginase protein expression and CAT 1 and 3 mRNA expression in irradiated lungs could be considered as molecular signs during the initial phase of radiation pneumonitis. Consistently with this assumption, decreased Arg I and II protein expression and CAT 1 and 3 mRNA expression in ALC pretreated group could be related to lower intensity of inflammatory reaction observed on a microscopic level.

CONCLUSION

This study shows that after high-dose local chest irradiation, ALC pretreatment significantly improves clinical prognosis and seems to reduce intensity of post-radiation changes on a molecular and microscopic level. With respect to its high tolerance, low toxicity and possible oral administration [26, 32], ALC supplementation may significantly contribute to force protection in radiation risk situations.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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