

EFFECTS OF PDT WITH 5-AMINOLEVULINIC ACID AND CHITOSAN ON WALKER CARCINOSARCOMA

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Porphyrins and new chitosan hydrogels based composites with porphyrins are used as active cytotoxic antitumor agents in photodynamic therapy (PDT). **Aim:** The present study evaluates the effects of photodynamic therapy (PDT) with 5-aminolevulinic acid (5-ALA) and 5-ALA associated with chitosan (CS) using Walker carcinosarcoma in rats as experimental model. **Methods:** The animals were irradiated with red light ($\lambda = 685$ nm, $D = 50$ J/cm², 15 min) 3 h after i.p. administration of 5-ALA (250 mg/kg b.w.) or a mixture of 5-ALA (250 mg/kg b.w.) and CS (1.5 mg/kg b.w.). The animals were sacrificed at 1, 3, 6, 24 h and 14 days after the treatment. The effects of PDT were investigated by morphological studies, monitoring the 5-ALA induced protoporphyrin IX (Pp IX) level in tumor tissue and serum, MMP 2 and 9 (gelatinases) activity in tumor and malondialdehyde level (MDA), marker of the lipoperoxidation process, in tumor and serum. **Results:** Zymography revealed an increased activity of MMP 2 in tumors from animals treated with 5-ALA PDT. PDT with 5-ALA induced a higher lipid peroxidation in tumor tissue compared with 5-ALA-CS. CS associated to 5 ALA PDT enhanced the accumulation of PS in tumors inducing earlier necrotic changes. In the same time CS reduced MMP 2 activity. **Conclusion:** Our results suggest that MMPs activation and oxygen reactive species are involved in PDT effects.

Key Words: chitosan, PDT, MMPs, malondialdehyde, protoporphyrin IX.

The ideal cancer treatment should both destroy the primary tumor and at the same time educate the immune system to recognize the tumor as foreign so that distant metastases will also be eradicated. PDT by its effects on tumor cells and on tumor vessels as well as by the activation of the host's immunity cells [1] is among the modern non-invasive methods of anti-tumor treatment.

PDT involves the administration of a photosensitizing agent (PS) and the exposure of the tumor to light with an adequate wavelength so that the photosensitizer would pass in an excited singlet [2]. From this state the PS can pass in a triplet state in which it interacts either with the molecular oxygen generating singlet oxygen (¹O₂) or with the biomolecules in the tissues producing radical forms of the PS. These reactive forms, by their reaction with oxygen, generate hydroxyl radical, hydrogen peroxide and superoxide anion [3, 4], which in their turn oxidize the macromolecules in the cells and compromise the cell function or even determine cell death by apoptosis or necrosis.

PDT also affects endothelial cells of tumor vessels resulting vascular damage [5]. In addition to this direct killing process, tumor eradication also arises from an acute inflammatory response featured by an increased level of various mediators in the PDT-treated tumor area (IL-1 β , G-CSF, IL- 8, MIP-2) [1].

The matrix metalloproteinases (MMPs) are zinc dependent endopeptidases involved in cell migration,

angiogenesis, tumor growth and metastasis, etc. [6]. MMPs are synthesized in a latent form and are transformed to biologically active proteases under the influence of some cytokines, growth factors, oncogenes or reactive oxygen species [7]. Evidence exist that MMP-2 and MMP-9 are involved in microvessel formation during the early phases of angiogenesis, but also in the reabsorption of neovascularisation, involution and regression of the vessels in the later stages [8].

In an attempt to find an ideal PS which would reach a maximum concentration in the tumor, would show a good light absorption and eliminate rapidly in order to avoid skin phototoxicity but would produce adequate amount of reactive oxygen species, different classes of PS were applied in PDT. PDT with 5-ALA offers promising results in this direction and it was approved in the therapy of superficial skin carcinoma, especially in basocellular carcinoma and in buccal cavity dysplasia [2].

Delta aminolevulinic acid or 5-ALA is not itself a PS but, after administration, it enters in the natural biosynthesis pathway of the porphyrins and is transformed to protoporphyrin IX (PpIX), a substance presenting destructive effect on tumor after irradiation. The preferential accumulation of 5-ALA in the tumor is accounted for the intense metabolic activity of tumor cells and to the limited capacity of ferrochelatase to convert porphyrins in heme [4].

Because the systemic administration of PS is limited by the reduced penetrability in the tumor tissue, an improvement of the therapeutic effect is attempted either by the use of different irradiation schedules [9], by the topical application or in liposome of the PS or by the use of some drug-carrier preparations ensuring the penetration and remanence of the substance in the tumor. In this connection, associated administration of

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Abbreviations used: 5-ALA – 5-aminolevulinic acid; CS – chitosan; MDA – malondialdehyde; MMP – matrix metalloproteinases; PDT – photodynamic therapy; PpIX – protoporphyrin IX, PS – photosensitizing agent; SDS – sodium dodecylsulfate.

5-ALA and CS, a cationic biocompatible polysaccharide derived from partial deacetylation of chitin, a natural product, is intended. Experimental data demonstrated that CS nanoparticles have cytotoxic effects on various tumor lines, both *in vivo* and *in vitro* [10, 11]. The mechanisms involved are related to the changes in the cell surface structure [12], to the decrease of the mitochondrial membrane potential [10] and to the induction of lipid peroxidation [12]. Additionally, an improvement of the host's immune response to the photodestructive effect on the tumor cells was found in experiments of photodynamic therapy using CS [13].

The present study aims at evaluating the effects of the photodynamic therapy with 5-ALA and CS on the activity of MMP 2 and 9 (gelatinases) and on the oxidative stress parameters using as experimental model Walker 256 carcinosarcoma in rats.

MATERIALS AND METHODS

Reagents. Five-aminolevulinic acid (purity 98%) was obtained from Sigma-Aldrich Inc. (Germany). Chitosan, with an acetylating degree 93.2% and a medium molecular weight (600 kDa) obtained from Sigma-Aldrich (Germany) in the form of 1–8 mm white-yellowish flakes was dissolved in 0.1% acetic acid in water, to obtain a final solution of 0.1%. Absolute ethanol and n-butanol were purchased from Chimopar (Bucharest). Protoporphyrin IX, KH_2PO_4 , sodium dodecylsulfate (SDS), 2-thiobarbituric acid, acetonitrile, Triton X-100 were obtained from Sigma-Aldrich Chemicals GmbH (Germany), EDTA- Na_2 from Merck KgaA Darmstadt (Germany). All the reagents were of analytical grade.

Animals and tumor. The study was performed on 90 Walker carcinosarcoma bearing male Wistar rats (180 ± 20 g, three months old). Wistar albino rats were obtained from the Animal Department of "Iuliu Hatieganu" University of Medicine and Pharmacy from Cluj-Napoca. They were kept for ten days with 12 h dark and 12 h light regimen in the Physiology Department in order to acclimatize. The animals were fed with a standard pellet diet and received water *ad libitum*. All the experiments were performed according to the approved animal-care protocols of the Ethical Committee on Animal Welfare of the "Iuliu Hatieganu" University. The rats were s.c. grafted with small fragments of Walker tumor in the right thigh. At this site the tumor was easily accessible to measurement with callipers and to the treatment. The photodynamic therapy was applied when the tumor reached 1 cm^3 (Ethic committee permission to work with animals).

PDT Protocol

Drug administration. For administration 5-ALA was dissolved just prior to use in PBS and the pH adjusted to 7 with 1N NaOH. The animals were injected i.p. with 5-ALA 250 mg/kg b.w. in 0.75 ml solution. All the solutions were protected from light with aluminium sheets. For the associated treatment of 5-ALA and CS, the CS solution was added to that of 5-ALA in PBS. The final mixture was also neutralized. The administered dose of CS was 0.15 mg/kg b.w. in 0.75 ml solution.

Light treatment. Three hours from drug administration the light treatment was applied. The animals were shaved on the right thigh and were anaesthetized with an i.p. injection of ketamine xylazine cocktail (90 mg kg^{-1} ketamine, 10 mg kg^{-1} xylazine). The irradiation was performed with red light applied directly on the skin above the tumor ($\lambda = 685 \text{ nm}$) in a dose of 50 J/cm^2 at a mean power of 25 W and a frequency of 10 Hz, for 15 min. The source of light used came from a Laser Therapeutic model D-68. At intervals of 1, 3, 6 and 24 h and after 14 days from irradiation groups of five animals were weighed, tumor diameters were measured and whole blood samples on EDTA were taken. After killing of rats by cervical dislocation the tumors were removed, fragmented, frozen and kept at -80°C . For the histopathologic examination two tumors/group were immersed in 10% formalin solution. For supplementary histopathologic examination one group was sacrificed after 96 h.

Determination of PpIX in the plasma and tumor.

Tumor fragments were homogenized for 2 min on ice in PBS (0.250 g tissue: 1 ml PBS) with Polytron homogenizer, centrifuged for 5 min at $3000 \times g$. Samples of plasma and tumour tissue (0.1 ml) were treated with a mixture of acetonitrile and absolute ethanol (1 : 7), vortexed for 1 min and centrifuged for 10 min at $5000 \times g$. Porphyrin was determined spectrofluorimetrically (excitation at 400 nm and emission at 626 nm) with a Perkin Elmer LS45B spectrofluorimeter [14]. The concentration of PpIX in the samples was calculated from standard curves and expressed as $\mu\text{moles/ml}$ or mg protein .

Evaluation of MMPs activity. The determination of the activity of gelatinases was performed by zymography [15]. The samples to be analysed were homogenized with Ultra Turrax in a medium with 2% glycerin and 2% SDS in 62.5 mM Tris-HCl buffer. After centrifugation, the protein in the supernatant was determined by a modification of Lowry's method. Five μg protein samples were subjected to electrophoresis in gels of 10% polyacrylamide copolymerized with 1 g/ml gelatin at 100 V for 2 h. After electrophoresis the gels were washed with 2.5% Triton X-100 solution for 1 h, then incubated for 18 h at 37°C in Tris-HCl 50 mM buffer, pH 7.4, containing 5 mM CaCl_2 , 200 mM NaCl and 0.02% BRIJ-35. Thereafter the gels were stained with Coomassie Brilliant Blue R-250 1% solution for 2 h, then discoloured in 10% acetic acid solution. The proteolytic activity appears as clear lytic white bands on an otherwise blue gel. The quantification of the activities was accomplished with a VILBER-LOURMET automatized image analyzer based on Bio 1 D program. The proteolytic activities were evaluated as against a weight marker confirmed with an enzymatic marker and expressed as arbitrary units/ $\mu\text{g protein}$.

Evaluation of the oxidative stress by determination of MDA. The tumor tissue fragments were homogenized using a Polytron homogenizer for 3 min on ice in 50 mM Tris buffer with 10 mM EDTA, pH 7.4, added in a ratio of 1 : 4 (w/v). The suspension was centrifuged for 5 min at $3000 \times g$ and at 4°C . The plasma or tumor homogenate samples were heated in a boiling

water bath for 1 h with a solution of 10 mM 2-thiobarbituric acid in 75 mM K_2HPO_4 , pH 3 solution. After cooling the reaction product was extracted in n-butanol. The MDA was spectrofluorimetrically determined in the organic phase using a synchronous technique with excitation at 534 nm and emission at 548 nm [16]. The MDA values are expressed as nmoles/ml (plasma) and nmoles/mg protein (tissue homogenate).

The histopathologic examination. For the histopathologic examination tumor fragments obtained after 6 h, 9 h, 24 h, 96 h and 14 days were fixed in 10% formalin solution. The sections were stained with haematoxylin and eosin and were examined under the light microscope with 4X and 10X objective.

Statistical analysis. The data were statistically analysed using the SPSS 13 program. For overall comparisons inside the same group X^2 test was used and in order to compare two different moments in a group Wilcoxon test was applied. For overall comparisons between two groups (control group non treated vs 5-ALA PDT and 5-ALA PDT vs 5-ALA-CS PDT) Mann–Whitney test was applied. $p < 0.05$ was considered significant.

RESULTS

Studies concerning PpIX pharmacokinetics in the plasma and tumor tissue. In all studied animals the concentration of PpIX in plasma and tumor was monitored at intervals of 1 h, 3 h, 6 h, 24 h and 14 days after PDT (Fig. 1). Regarding the plasma concentration of PpIX, a significant difference was noted between the groups treated with 5-ALA PDT and the groups with associated treatment (5-ALA-CS). The plasma level of PpIX in 5-ALA PDT increased 1 h after the therapy and it was maintained high for 6 h, with a peak at 3 h. In the presence of CS the concentration of PpIX had significantly higher levels at 1 h ($p < 0.01$). After 6 h PpIX disappeared rapidly from the plasma to nearly undetectable values ($p < 0.001$).

One hour and 3 h after the administration of 5-ALA and CS the concentration of PpIX in the tumor tissue was increased as compared with those in the groups treated with 5-ALA only (1 h: 0.016 ± 0.010 vs 0.004 ± 0.004 μ moles/mg protein; 3 h: 0.005 ± 0.004 vs 0.001 ± 0.002 μ moles/mg protein; $p < 0.01$). Six hours after PDT PpIX level decreased to zero in the tumor from animals in both groups. Therefore it can be asserted that CS enhances the accumulation of PS in the tumor. However CS does not influence the time this persists in the tumor tissue.

Comparing the ratio (R) between PpIX level in the tumor and plasma at various intervals in the same group, the predominant concentration of PpIX can be found in the tumor in the first hour after PDT (5-ALA PDT: $R = 0.007$; 5-ALA-CS PDT: $R = 0.018$). The ratio decreased after 3 h (5-ALA PDT: $R = 0.0023$; 5-ALA-CS PDT: $R = 0.0071$). The comparison of the same ratio in

animals treated with 5-ALA PDT and those with associated CS revealed statistically significant differences after 1 h ($R = 0.007$ vs $R = 0.018$) and 3 h ($R = 0.0023$ vs $R = 0.071$). The highest tumor/plasma PpIX ratio was noted in the groups with associated CS in the first hour after PDT, suggesting a greater concentration of the active substance in the tumor tissue immediately after PDT.

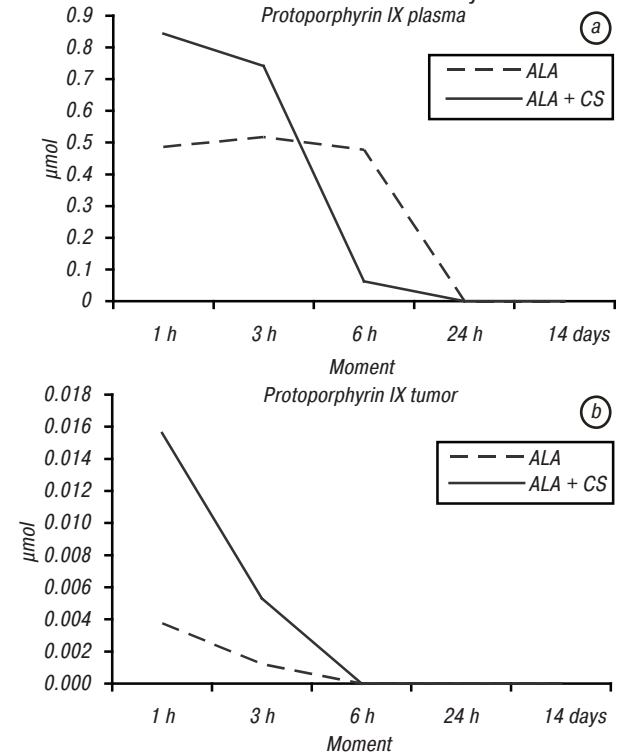


Fig. 1. Concentrations of PpIX in the plasma (a) and tumor (b) of Walker carcinosarcoma bearing rats at 1 h, 3 h, 6 h, 24 h and 14 days after ALA PDT and ALA-CS PDT

MMPs activity in the tumor tissue. Analysis of zymographic images revealed the presence of MMP 2 in all studied groups (Table). After 1 h 5-ALA PDT increased MMP 2 activity in tumor as compared with those in the untreated controls. During the next intervals, the activity of MMP 2 decreased although to higher levels than the corresponding ones in the group treated with CS (Fig. 2). PDT with 5-ALA-CS modified the behaviour of MMP 2. A significantly reduced activity of the pro-enzyme form of MMP 2 was found as compared with the 5-ALA PDT group one hour after the treatment. Neither the mature nor the proenzyme forms of MMP 9 were detected in analysed specimens.

The study of oxidative stress. Photodynamic therapy induced lipid peroxidation in the tumor tissue still in the first hours from the initiation of the treatment. MDA concentration rose significantly following 5-ALA PDT as compared with the untreated group, reaching the highest levels 24 h after the treatment (0.75 ± 0.22 vs 0.35 ± 0.05 μ moles/mg protein; $p < 0.05$) (Fig. 3). Fourteen days after the treatment the level of MDA was low (0.28 ± 0.20 μ moles/mg protein).

Table. MMP2 activities (arbitrary units/ μ g protein) in tumor tissue from ALA PDT respectively ALA-CS PDT treated animals

	Control group	ALA 1 h	ALA 6 h	ALA 24 h	ALA 14 days	ALA + CS 1 h	ALA + CS 3 h	ALA + CS 6 h	ALA + CS 24 h	CS 24 h
Pro MMP 2	1178.9 \pm	1854.5 \pm	779.5 \pm	1612.5 \pm	1369.6 \pm	973.8 \pm	1362.9 \pm	1532.95 \pm	1188.2 \pm	1453.4 \pm
	184.5	59.5*	90.5	142.2**	220.1	83.8***	72.7	127.2	11****	34.1
MMP 2	528.4 \pm	1025 \pm 23	641.5 \pm	691 \pm 16.4	616 \pm 184.8	744.7 \pm	481.5 \pm	879.8 \pm	589.6 \pm	593.4 \pm
	307.2		28.4			129.8	61.6	68.5	14.6	41.4

Notes: * $p < 0.01$ vs control; ** $p < 0.05$ vs control; *** $p < 0.001$ vs ALA 1 h; **** $p < 0.05$ vs ALA 24 h.

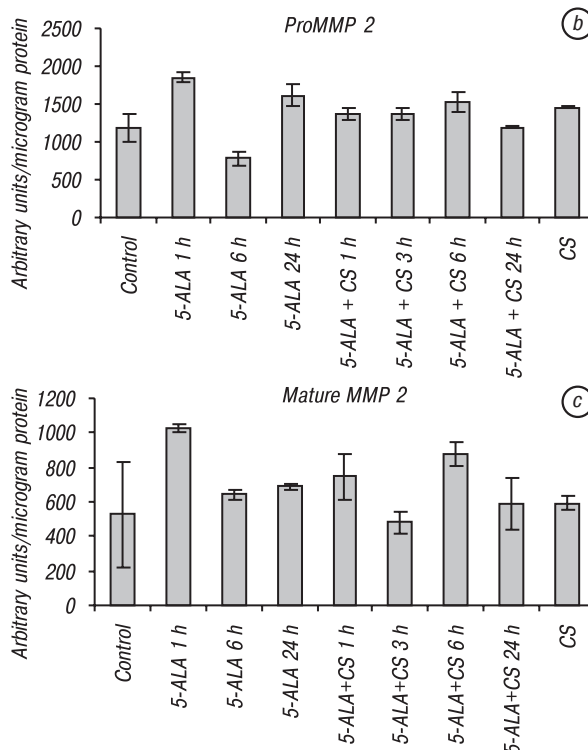
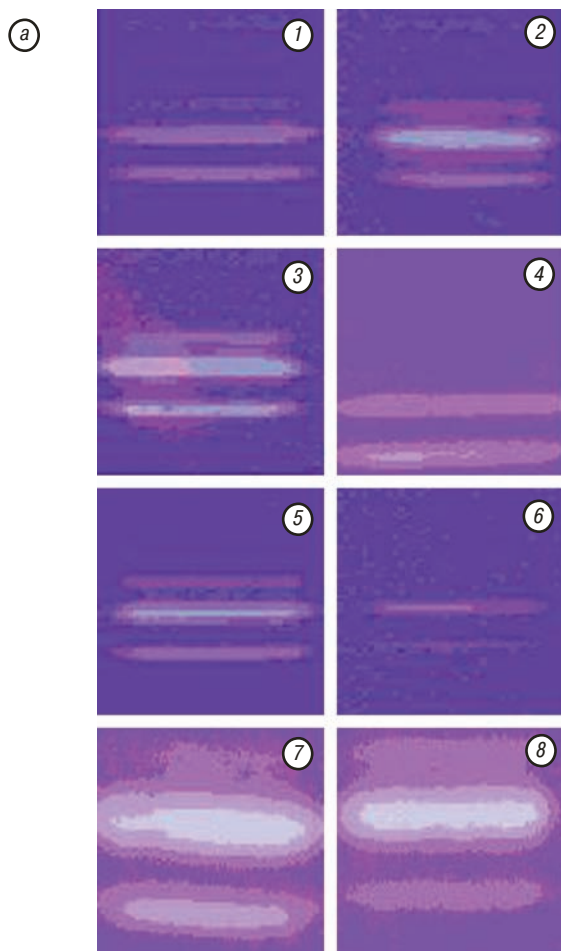


Fig. 2. ALA PDT induces activation of matrix metalloproteinase 2 (MMP 2) in Walker tumors. *a* – gelatin zymography documenting the presence of MMP 2 in tissue lysates from ALA PDT and ALA-CS PDT-treated tumors (1 – control group; 2 – CS 24 h; 3 – 5-ALA 1 h; 4 – 5-ALA 6 h; 5 – 5-ALA 24 h; 6 – 5-ALA 14 days; 7 – 5-ALA-CS 1 h; 8 – 5-ALA-CS 24 h); *b* and *c*, proMMP 2 and MMP 2 mature activities (arbitrary units/ μ g protein) in tumor tissue from ALA PDT respectively ALA-CS PDT treated animals

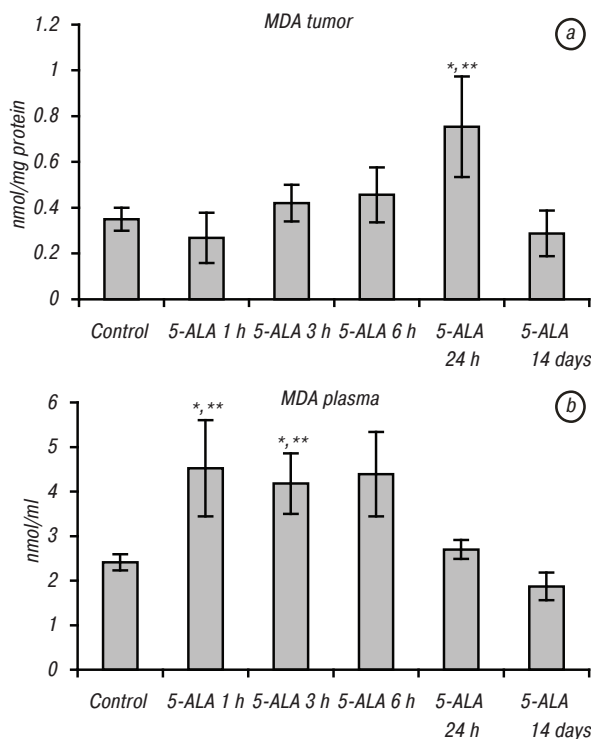


Fig. 3. Malondialdehyde levels in tumor tissues (*a*) and plasma (*b*) of Walker carcinosarcoma bearing rats after 1 (5-ALA 1 h), 3 (5-ALA 3 h), 6 (5-ALA 6 h), 24 (5-ALA 24 h) hours and 14 days (5-ALA 14 days) from photodynamic therapy with 5-ALA *a* – *significant ($p < 0.05$) vs controls and 5-ALA 3 h; **significant ($p < 0.01$) vs 5-ALA 14 days. *b* – *significant ($p < 0.05$) vs controls and 5-ALA 24 h; **significant ($p < 0.01$) vs 5-ALA 14 days.

The association of CS to 5-ALA PDT significantly reduced the formation of lipid peroxides in the tumor after all tested intervals (1h: 0.13 ± 0.02 μ moles/mg protein, $p < 0.05$; 3 h: 0.15 ± 0.02 μ moles/mg protein; 6 h: 0.15 ± 0.02 μ moles/mg protein; $p < 0.01$; 24 h: 0.13 ± 0.04 ; $p < 0.001$) (Fig. 4). The evolution of the same parameter in plasma faithfully follows its evolution in the tumor. The plasma MDA level in the groups treated with 5-ALA PDT has the highest value at 1 h interval (4.52 ± 1.08 μ moles/ml) and remains high at 3 h and 6 h (4.18 ± 0.68 and 4.39 ± 0.95 μ moles/ml respectively). After 24 h and 14 days MDA was significantly lower (2.70 ± 0.21 μ moles/ml; 1.87 ± 0.31 vs 2.41 ± 0.18 μ moles/ml) (see Fig. 3). The association of CS to 5-ALA PDT lowers the plasma concentration of MDA (1 h: 3.62 ± 0.57 μ moles/ml; 3 h: 2.46 ± 0.34 μ moles/ml, $p < 0.01$; 6 h: 2.80 ± 0.61 μ moles/ml, $p < 0.001$; 24 h: 2.49 ± 0.40 μ moles/ml) (Fig. 4).

Histologic studies. The tumor sections stained with haematoxylin and eosin (HE) were examined under the light microscope at various intervals following PDT in order to detect the onset of intratumor necrosis. Six hours after 5-ALA-PDT oedema, vascular congestion and profuse leukocytic inflammatory infiltration occurred in the intratumor septa (Fig. 5). At 9 h isolated presence of amorphous cells with pyknotic or absent nuclei, without clear intercellular limits, interspersed with viable cell areas was noted. The changes were more consistent, large areas of necrotic cells predominantly in the centre of the tumor were observed after 96 h. Two

weeks after PDT the necrosis was extended in the entire tumor section. The administration of CS associated with PDT induced an early agglomeration of inflammatory cells in the tumor septa. At 24 h after 5-ALA-CS PDT necrotic cells occurred in the centre of the tumor.

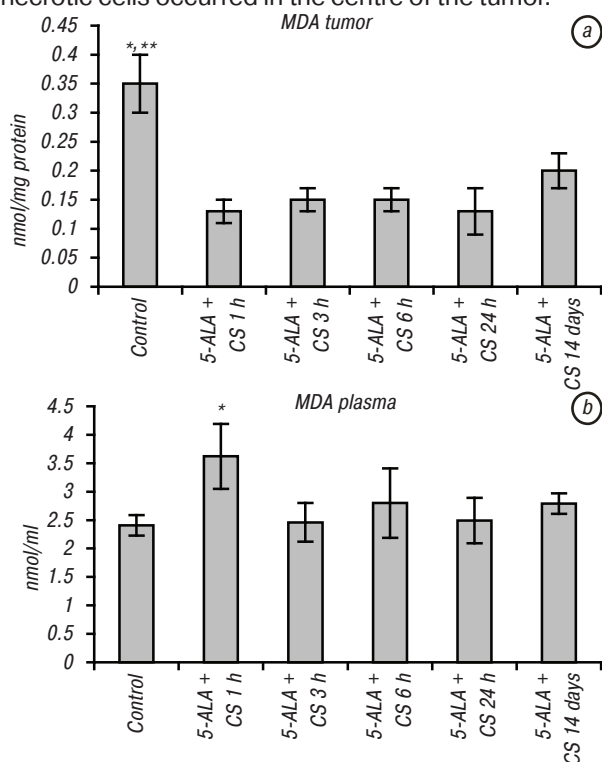


Fig. 4. Malondialdehyde levels in tumor tissues (a) and plasma (b) of Walker carcinosarcoma bearing rats after 1 (5-ALA-CS 1 h), 3 (5-ALA-CS 3 h), 6 (5-ALA-CS 6 h), 24 (5-ALA-CS 24 h) hours and 14 days (5-ALA-CS 14 d) from photodynamic therapy with 5-ALA and chitosan (CS)

a — *significant ($p < 0.05$) vs control; **significant ($p < 0.01$) vs control; b — *significant ($p < 0.01$) vs control.

DISCUSSION

Photodynamic therapy depends on the delivery of a PS to the target tissue that, under light exposure, produces singlet oxygen and other reactive oxygen species, which in turn cause the death of the treated cell. The association of CS to PDT, the so-called laser/sensitizer assisted immunotherapy, offers additional advantages, namely it allows the easier penetration and the concentration of the PS in the tumor and stimulates the host's immune response, with long-term antitumor effect without any secondary effects [13, 17, 18]. The present investigation started from the idea to optimize PDT by combining 5-ALA, a compound with doubtless antitumor effects, with a natural biopolymer showing cytotoxic, immunoadjuvant and drug-carrier properties. The study of the pharmacokinetics of PpIX clearly demonstrated that the association of CS to 5-ALA PDT ensures high concentrations of the PS in the tumor and plasma. These high levels of PS probably enhance the PDT efficiency. The data in the present study evidenced that 5-ALA PDT in a dose of 250 mg/kg b.w. and at a fluency of 50 J/cm² induced cell necrosis in the tumor and, consequently tumor destruction. The results regarding lipid peroxidation in animal tumors and plasma suggest the involvement of reactive oxygen species the mecha-

nisms leading to tumor cells killing as a consequence of PDT. Thus, the MDA level raised significantly 3 h after the treatment, had a maximum at 24 h, and then decreased down to the level of the control group. This evolution of MDA is correlated with the histological changes noted after the treatment.

CS inhibitory effects on the proliferation of the tumor cells and not in the last turn the activation of the leukocytes, of the cellular and humoral immunity recommend it as an ideal compound with immunoadjuvant antitumor effect [10, 13, 17]. CS increased the curative effect as compared with The Complete Freund Adjuvant, with Incomplete Freund Adjuvant and *Corynebacterium Parvum* on tumors treated with PDT [19]. There are studies that demonstrate that the antitumor effect of CS is related to the induction of tumor cell apoptosis and to the action on the tumor vessels [20]. Moreover, CS determined the activation of the natural killer cells and of chemotaxia [21]. The administration of CS microspheres exerted toxic effects on cell lines of murine melanoma B16F10 and on tumor cells of human gastric carcinoma MGC803 [22, 23].

In this study the i.p. administration of CS with 5-ALA, followed by irradiation, determined the accumulation of inflammatory cells in the septa of the tumor immediately after the institution of PDT and induced necrosis after 24 h.

After all tested intervals, the combined therapy with CS and 5-ALA lowered the lipid peroxidation below the level of that determined by the administration of 5-ALA PDT only, significant results being obtained after 3 h, 6 h and 24 h ($p < 0.01$), suggesting an antioxidant effect of CS.

The results are in agreement with these obtained by other investigators. In this connexion, Jeon reported a reduction in thiobarbituric reactive substances (TBARS) levels and an increase of antioxidant enzymes activities after CS administration in animals [24]. Additionally ESR studies and 1 diphenyl-2-picrylhydrazyl radical (DPPH) test for antioxidant activity suggested the scavenger properties of CS for hydroxyl radical and superoxide anion [25, 26]. These effects are related to the chemical structure of CS [27].

MMPs are involved in tumor angiogenesis, growth, invasion and metastatic potential. In solid tumors MMPs are often expressed by stromal cells and macrophages rather by tumor cells. Increased MMPs activities were reported after PDT [7]. Modulation of MMPs activities with different agents combining antiangiogenic drugs with PDT show improved treatment responsiveness [7]. In our experimental model, gelatin zymography on tumor homogenates revealed the induction of enzymatically active form of MMP2 after 5-ALA PDT. The association of CS to 5-ALA PDT reduced the activity of MMP 2 in the first hour after the treatment. However, contradicting data were reported in the literature concerning the effects of CS on the activity and expression of MMPs. These differences are probably related to the type of CS and to the experimental models used [7, 28]. Recent investigations

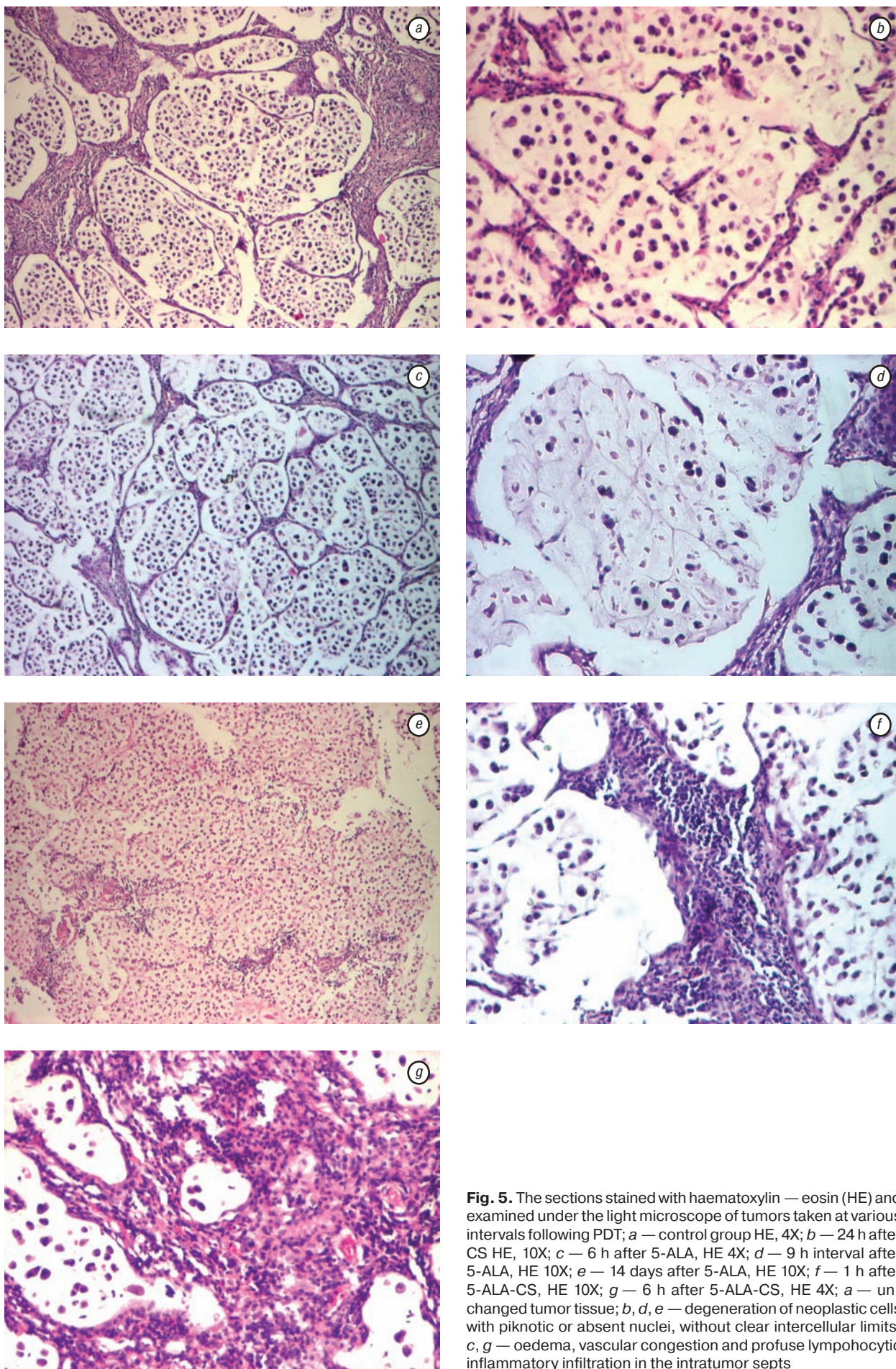


Fig. 5. The sections stained with haematoxylin — eosin (HE) and examined under the light microscope of tumors taken at various intervals following PDT; a — control group HE, 4X; b — 24 h after CS HE, 10X; c — 6 h after 5-ALA, HE 4X; d — 9 h interval after 5-ALA, HE 10X; e — 14 days after 5-ALA, HE 10X; f — 1 h after 5-ALA-CS, HE 10X; g — 6 h after 5-ALA-CS, HE 4X; a — unchanged tumor tissue; b, d, e — degeneration of neoplastic cells with piknotic or absent nuclei, without clear intercellular limits; c, g — oedema, vascular congestion and profuse lymphocytic inflammatory infiltration in the intratumor septa

found that CS inhibits the expression of MMP 2 on human fibroblasts and of MMP 9 on human fibrosarcoma respectively [29]. This effect seems to be the result of the expression and activation of MMP 2 [30]. Moreover, it was noted that CS inhibits the expression of the transcription factor AP-1 [31] without an effect on MMPs inhibitors (TIMP-1) or on NF- κ B. Other studies showed that CS administration increases the release of MMPs in the first hours after the treatment [32]. Of course, the effects of CS are dependent on physico-chemical properties of the CS used (acetylating degree, particle size, way of administration), on tumor cells used, techniques for the evaluation of the effects of CS, etc.

CONCLUSIONS

Present investigation revealed major histologic alterations in the tumor tissue with extended areas of necrosis after 5 ALA PDT. These changes are correlated with high levels of MDA, a marker of the presence of reactive oxygen species in plasma and tumor. Additionally, in our experimental model PDT determined the activation of MMP 2.

CS associated to 5 ALA PDT enhanced the accumulation of PS in tumors inducing earlier necrotic changes. In the same time CS reduced MMP 2 activity. Complex mechanisms are probably involved in these processes, which deserve further investigations.

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ДЕЙСТВИЕ PDT, 5-АМИНОЛЕВУЛЕНОВОЙ КИСЛОТЫ И ХИТОЗАНА НА КАРЦИНОСАРКОМУ УОКЕРА

Порфирины и новые соединения, основу которых составляют гидрогели хитозана с порфиринами, используются как активные цитотоксические противоопухолевые препараты при фотодинамической терапии (PDT). *Цель:* оценить действие PDT с 5-аминолевуленовой кислотой (5-ALA) и 5-ALA, ассоциированной с хитозаном (CS), на клетки карциносаркомы Уокера. *Методы:* крыс облучали красным светом ($\lambda = 685$ нм, $D = 50$ Дж/см², 15 мин) 3 ч после внутрибрюшинного введения 5-ALA (250 мг/кг) или смеси 5-ALA (250 мг/кг) и CS (1,5 мг/кг). Подопытных животных забивали через 1 ч, 3 ч, 6 ч, 24 ч и 14 дней после воздействия PDT. Эффект PDT определяли с помощью морфологических исследований, регистрируя уровень протопорфина IX (Pp IX), вызываемого 5-ALA, в опухолевой ткани и сыворотке крови, активность MMP 2 и 9 (желатиназы) в опухоли и уровень малонового диальдегида (MDA), маркера процесса перекисного окисления липидов, в опухоли и сыворотке крови. *Результаты:* зимографические исследования показали повышенную активность MMP 2 в опухолях животных, которых подвергали 5-ALA PDT. PDT с 5-ALA вызывала повышенный уровень перекисного окисления липидов в опухолевой ткани по сравнению с 5-ALA-CS. CS с 5-ALA PDT усиливал накопление фотосенсибилизирующего вещества (PS) в опухолях, вызывая более ранние некротические изменения. В то же время CS снижал активность MMP 2. *Выводы:* полученные результаты позволяют предположить, что для проявления эффектов PDT необходимы активация MMP и образование активных форм кислорода.

Ключевые слова: хитозан, PDT, MMP, малоновый диальдегид, протопорфин IX.