EXPERIMENTAL STUDY OF INCREASING OXIDATIVE STRESS IN PDT USING CHIROXY

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REZUMAT


Cuvinte cheie: Chiroxy, stres oxidativ, terapie fotodinamică, substanță fotosensibilă, markeri de stres

ABSTRACT

Introduction: Precursors of porphyrines are used in photodynamic therapy (PDT) and induce tumor necrosis and cellular apoptosis by two main mechanisms: type I (reaction with cells) and type II (energy transfer to oxygen and generation of singlet oxygen). Chiroxy oxygenating skin cream is designed to increase the oxygen content of skin by delivering O2 via nanosomes. Objective: The present study evaluates the effects of PDT with 5 ALA (5 aminolevulinic acid) and 5 ALA with an increasing oxygen level substance (Chiroxy), using Walker carcinosarcoma bearing rats as experimental model. Material and methods: The rats’ tumors were irradiated by red light (λ = 685 nm, dose = 50 J/cm², 15 min) 3 h after topical administration of 5 ALA 20% or a mixture of 5 ALA 20% and Chiroxy. The animals were sacrificed after 24 hours. The effects of PDT were investigated by morphological studies, monitoring serum level and tumor level of malondialdehyde (MDA) and protein carbonyls (PC) as oxidative stress markers, glutathione and thiol group (–SH) for appreciation of antioxidant defense. Results: Increased tumor levels of MDA, PC and –SH on mice treated only with Chiroxy prove that the substance increases the oxygen level of the cells and oxidative stress. The association of 5 ALA to PDT increases its oxidative effect. Increased serum level of glutathion shows an adaptation to oxidative stress induced by PDT. Conclusion: Results show that adding an oxygenating substance to a photosensitizer enhances the oxidation of macromolecules and the therapeutic effects.

Key Words: Chiroxy, oxidative stress, photodynamic therapy, photosensitizer, stress markers

INTRODUCTION

Photodynamic treatment (PDT) is an emerging therapeutic procedure for the management of cancer and some precancerous skin diseases. It consists of interaction of a photosensitizer (PS), light with appropriate wavelength and oxygen. The combination of photosensitizer and light in presence of oxygen generates singlet oxygen and other cytotoxic reactive oxygen species (ROS), such as superoxide anions and...
hydroxyl radicals. Oxygen radicals are continuously generated within mammalian cells as a consequence of the use of oxygen in aerobic respiration. Photosensitizers exposed to light generate singlet oxygen which can induce cell destruction in vitro and in vivo. These specific sub-cellular targets of PDT (which can differ for various sensitizers) are determinants for activation after oxidative stress.\(^1\)

Singlet oxygen generated by the type II pathway (mediated by an energy transfer process with ground state oxygen) has a central role in photodynamic cytotoxicity because of the highly efficient interaction of the singlet biomolecules.\(^2\) Lipid peroxidation and protein cross-linking affect the depolarization and inactivate membranous enzymes. The increased membrane permeability inhibits transport of aminoacids and nucleosides.

The response to photosensitization includes the activation of gene encoding for several stress proteins such as heat shock proteins, heme oxygenase and glucose-regulated proteins as well as a transient induction of the early response genes (e.g: c-fos, c-jun, c-myc).\(^3\)

The cells possess endogenous defense mechanisms, such as antioxidative enzymes (superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic antioxidative molecules (vitamin E, vitamin C, glutathione), protecting it from free radicals by reducing and neutralizing them.

PDT with 5 ALA was approved in the therapy of superficial skin carcinoma, especially in basal cell carcinoma, because its special property to reach a maximum concentration in the tumor, its good light absorption and quick elimination from skin in order to avoid skin toxicity while producing adequate amount of reactive oxygen species.\(^4\)

Delta aminolevulinic acid or 5–ALA is not itself a PS but after administration enters in the natural biosynthesis pathway of the porphyrins, is transformed to protoporphyrins and then to protoporphyrin IX (PpIX), which has a destructive effect on tumor cells after irradiation.

Chiroxy is an oxygenating substance which increases the partial pressure of oxygen of the skin thanks to the oxygen encapsulated in nanosomes. This modern technology enables optimum penetration of the oxygen into the skin right up to the subcutaneous tissues.\(^5\)

**AIM OF THE STUDY**

The present research aims to evaluate the effects of PDT with Chiroxy added to 5-ALA and oxidative stress parameters using as experimental model Walker 256 carcinoma in rats.

**MATERIAL AND METHODS**

**Reagents**

5-aminolevulinic acid (purity 98%) was obtained from Sigma-Aldrich Inc (Germany). Absolute ethanol and n-butanol were purchased from Chimopar (Bucharest). O-phtalaldehyde, 2,4 dinitrophenylhydrazine, trichloracetic acid, guanidine chlorhydrate, 2,2-dithiobisnitrobenzoic acid, 2-thiobarbituric acid were obtained from Sigma-Aldrich Chemicals GmbH (Germany), EDTA–Na\(_2\) from Merk KgaA Damstadt (Germany). All the reagents were of analytical grade.

**Animals and tumor**

The study was performed on 25 Walker carcinosarcoma bearing male Wistar rats (180 ± 20g, three months old). Wistar albino rats were obtained from the Animal Department of Iuliu Hatieganu University of Medicine and Pharmacy 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 subcutaneously grafted with small fragments of Walker tumor in the right thigh. At this site the tumor was easily accessible to measurement with calipers 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**

Four groups were created:
- group treated with Base III (vehicle) – 6 animals
- group treated with 5 ALA 20% - 6 animals
- group treated with 5 ALA 20% and Chiroxy – 7 animals
- group treated with Chiroxy only – 6 animals

For administration 5-aminolevulinic acid was included in Chiroxy for a group and in Base III (as vehicle) for another group. The animals were epilated a day before the experiment and 2-3 mg of the substances were topically applied on a 2 cm\(^2\) skin area the next day. The animals were covered by aluminium slide to be maintained on dark.

**Light treatment**

Three hours after the topical application of the substances the light treatment was done. The animals were anaesthetized with an intraperitoneal injection of ketamine xylazine cocktail (90 mg/kg body ketamine, 10 mg/kg body xylazine). The irradiation was performed...
with red light applied directly on the skin above the tumor ($\lambda = 685$ nm) in a dose of 50 J/cm$^2$ at a mean power of 25 Watt, at frequency of 10 Hz for 15 minutes. The light source came from a Laser Therapeutic model D-68. At 24 hours intervals the animals were weighed, tumor diameters measured and whole blood samples on EDTA were taken. The rats were sacrificed by cervical dislocation and then the tumors were removed, fragmented, frozen and kept at -80°C.

**Evaluation of the oxidative stress by the determination of MDA**

The fragments of the tumor tissue were homogenized using a Polytron homogenizer for 3 minutes on ice in 50 mM Tris buffer with 10mM EDTA, pH 7.4, added in a ratio of 1:4 (w/v). The suspension was centrifuged for 5 min at 3000 x g and 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$_2$HPO$_4$ pH 3 solution. The reaction product was extracted in n-butanol after cooling. The MDA was spectrofluorometrically determined in the organic phase using a synchronous technique with excitation at 534 nm and emission at 548 nm. The MDA values are expressed as nmol/ml (plasma) and nmol/mg protein (tissue homogenate).

**Determination of glutathione**

The measurement of glutathione through fluorimetric method is possible because of its property to make a fluorescent reaction with o-phtalaldehyde. Determinations are done in serum and homogenated tissues.

A volume of plasma was mixed with acid trichloracetic (TCA) 10% and after 10 minutes was centrifuged. The supernatant was separated and 1.7 ml phosphate buffer with pH 8 and 1 ml of o-phtalaldehyde were added. After 15 min the intensity of emission at 420 nm on an excitation of 350 nm was measured. Glutathione concentration is measured using a calibration curve realized with known concentrations of glutathione obtained in the same way. Concentration values are expressed in nmol/ml.

**Determination of protein carbonyl**

Serum samples were treated with 2.4 dinitrophenylhydrazine 10 Mm in HCl 2.5 N for 1 hour at room temperature and dark. After treatment with trichloracetic acid- 20% and separation by centrifugation, the precipitate was washed three times with a mixture of ethylic acetate and absolute ethylic alcohol 1:1(v/v). Subsequently, protein precipitate was dissolved in guanidin chloride 6M. Protein concentrate was determined on these samples by measuring the extinction at 280 nm and at 355nm (hydrazine absorption wavelength). Carbonyl concentration was calculated with formula $C = Abs_{355}$ x4.54nmol/ml. Final results were expressed in nmol/mg protein.

**Determination of thiol groups**

A plasma volume was mixed with Tris tampon (0.25M) - EDTA 20mM pH 8.2 and the absorbent was read at 412 nm. After 15 minutes 2,2-dithiobisnitrobenzoic acid (Ellman's reagent – DNTB) 10 mM reactive was added and the sample was read again with new yellowish absorbent on the same wavelength. The following formula was used:

$$(A_1 - A_2 - B) \times 1.57 \text{ mM/ml},$$

where:
- $A_1$ is the absorbent before adding DNTB;
- $A_2$ is the absorbent after adding DNTB;
- $B$ is the absorbent of blank sample with tampon;
- 1.57 is a factor from dilutions.

**Statistical analysis**

The data were statistically analyzed using a nonparametric Kruskall-Wallis test for global groups’ comparison and Mann-Whitney U Test for the comparison of two groups.

**RESULTS**

**General statistic results**

Statistic data obtained in this study are shown in Tables 1 and 2.

**Table 1. Data of investigated oxidative stress markers in serum**

<table>
<thead>
<tr>
<th>Serum</th>
<th>MDA nmol/ml</th>
<th>PC nmol/mg protein</th>
<th>SH mmol/L</th>
<th>GSH μmol/L (glutathion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group treated with Chiroxy</td>
<td>0.87±0.22</td>
<td>1.08±0.17</td>
<td>0.07±0.01</td>
<td>13.22±11.25</td>
</tr>
<tr>
<td>Group treated with 5 ALA 20% in Base III</td>
<td>1.84±0.89</td>
<td>0.80±0.18</td>
<td>0.107±0.04</td>
<td>6.69±4.14</td>
</tr>
<tr>
<td>Group treated with 5 ALA 20% in Chiroxy</td>
<td>1.01±0.35</td>
<td>0.83±0.30</td>
<td>0.06±0.03</td>
<td>9.72±5.45</td>
</tr>
<tr>
<td>Group treated with 5 ALA 20% in Base III</td>
<td>1.48±0.08</td>
<td>0.61±0.07</td>
<td>0.06±0.04</td>
<td></td>
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</tbody>
</table>

**Study concerning the levels of PC (protein carbonyl) in serum and tumor**

Kruskall-Wallis statistic test shows significant differences between all studied groups about protein
carbonyls levels in serum and tumor. In serum, statistic data are (PC nmol/mg protein): group treated with Chiroxy 1.08±0.17, group treated with 5 ALA 20% in Base III 0.80±0.18, group treated with 5 ALA 20% in Chiroxy 0.83±0.30, group treated with Base III 0.61±0.07 and p=0.05. (Fig. 1) In tumor, statistic data are (PC nmol/mg protein): group treated with Chiroxy 3.95±0.40, group treated with 5 ALA 20% in Base III 5.31±2.72, group treated with 5 ALA 20% in Chiroxy 5.03±2.31, and group treated with Base III: 1.54±0.47, with p=0.02. (Fig. 2)

Table 2. Data of investigated oxidative stress markers in tumor.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>MDA nmol/mg protein</th>
<th>PC nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group treated with Chiroxy</td>
<td>0.14±0.06</td>
<td>3.95±0.40</td>
</tr>
<tr>
<td>Group treated with 5 ALA 20%+ Base III</td>
<td>0.13±0.05</td>
<td>5.31±2.72</td>
</tr>
<tr>
<td>Group treated with 5 ALA 20% in Chiroxy</td>
<td>0.16±0.08</td>
<td>5.03±2.31</td>
</tr>
<tr>
<td>Group treated with Base III</td>
<td>0.14±0.08</td>
<td>1.54±0.47</td>
</tr>
</tbody>
</table>

Applying Man Whitney test and comparing groups on pairs, statistically significant differences are found about protein carbonyls in serum and tumor. Statistic data show significant differences of PC serum levels for the group treated with Chiroxy and irradiated, compared to the group treated with Base III and irradiated, with p = 0.01 and of PC tumor levels for same groups with p = 0.01. (Figs. 3, 4).

In addition, is increased the level of protein carbonyls in serum after applying Chiroxy in comparison with group treated with standard PDT with 5 ALA 20% in Base III vehicle (p=0.04). (Fig. 5)

In tumor tissue homogenate is observed a statistically significant increase of PC after treatment with Chiroxy (p=0.01) in comparison with control group treated with Base III. A similar result is seen in group treated after PDT with 5-ALA 20% in Base III (vehicle) (p=0.01) or 5-ALA in Chiroxy (p=0.01 versus Base III). Applying Chiroxy increases levels of PC in same manner as PDT with 5-ALA.

Study concerning the serum and tumor level of MDA (malondialdehyde)

On global statistic data concerning MDA (nmol/
ml) evolution are observed important differences in serum for all groups: group treated with Chiroxy 0.87±0.22, group treated with 5 ALA 20% in Base III 1.84±0.89, group treated with 5 ALA 20% in Chiroxy 1.01±0.35, group treated with Base III 1.48±0.08 and p=0.07.

MDA levels are lower in serum for the group treated with Chiroxy (p=0.02 versus Base III) and in the group treated with Chiroxy (p=0.05 versus the group treated with PDT 5 ALA 20% in Base III). (Fig.6)

Concerning overall data for MDA (nmol/mg protein) evolution in tumor, there are no significant differences for the following groups: group treated with Chiroxy 0.14±0.06, group treated with 5 ALA 20% in Base III 0.13±0.05, group treated with 5 ALA 20% in Chiroxy 0.16±0.08, group treated with Base III 0.14±0.08.

In the tumor there is no statistically significant differences between groups (p=0.53). (Figs.7-9).

Gluathione and thiol group are studied for appreciation of antioxidant defense capacity of cellular system. In the present study both of them did not change. The groups treated with PDT 5-ALA 20% in Base III and PDT 5-ALA 20% in Chiroxy did not present an increase in the response of antioxidant capacity (p=0.05).
Morphological Studies

The tumor sections stained with hematoxylin and eosin (HE) were examined under light microscope following PDT in order to detect the onset of intratumor necrosis. At 24 h isolated presence of amorphous cells with pyknotic or absent nuclei, without clear intercellular limits, interspersed with viable cell areas was noted. These aspects prove cytotoxic effects of PDT on tumor cells. (Figs. 10-13)

Figure 10. Morphological aspect of tumor treated with Base III, 100 X HE.

Figure 11. Morphological aspect of tumor treated with Chiroxy, 100X HE.

Figure 12. Morphological aspect of tumor treated with 5 ALA 20% in Base III, 40X HE.

Figure 13. Morphological aspect of tumor treated with 5 ALA 20% in Chirox, 40 X, HE.

DISSCUSIONS

The availability of molecular oxygen is extremely important for the sensitivity of tumor cells to photodynamic therapy. Oxygen plays a direct role in photochemical reactions producing the highly cytotoxic singlet oxygen. As far as PDT requires molecular oxygen during illumination to generate singlet oxygen, it is supposed an enhanced cytotoxicity of the treatment is obtained by increasing the amount of oxygen available for the photochemical reactions.

Astrid Hjelde et al. performed a study that investigated the effect of hyperoxia on PDT and lipid peroxidation in three different cancer cells lines.\textsuperscript{10} In this study they increased the oxygen level by raising its partial pressure in tumor cells after PDT treatment and measured cell survival under normoxia and hyperoxia, effect of hyperoxia and lipid peroxidation. They concluded there was no difference in cell survival between PDT under normoxia (21 kPa $\text{O}_2$) and PDT under any level of hyperoxia (100, 200, 300 or 400 kPa $\text{O}_2$). Increasing the pressure of oxygen did not enhance the photo-killing effect of the treatment but an improved outcome of PDT is obtained in combination with hyperbaric oxygen. These discrepancies can be explained by different demand for oxygen in the target cell or tissue. The presence of hypoxic cells in tumors is believed to be a major factor in limiting the effectiveness of PDT.

In our study Chiroxy was used with purpose of increasing oxygen molecular levels tumor cells and compared oxidative stress in four situations: PDT with 5-ALA 20%, PDT with 5-ALA 20% and Chiroxy, tumors treated with Chiroxy only and irradiated with red light an same wavelength as in PDT, and one control group treated with in Base III and irradiated with red light. Because ROS mediate PDT effects we chose
oxidative stress parameters as markers for appreciation of therapeutic efficiency. Malondialdehyde is a major end-point product of lipoperoxidation and is also used to evaluate oxidative stress. Cellular proteins are also targets for toxicity mediated by ROS which determine modifications of the side chains of aminoacids; breaking and cross linking of the chains. Daicoviciu et al. shows in a study that PDT with 5-ALA determines increased levels of lipid peroxides and protein carbonyls both in plasma and tumors in Walker 256 carcinoma bearing rats.  

Filip et al. estimated that MDA levels in tumor increased after PDT 5-ALA at 3 hours after the treatment, had a maximum at 24 hours, 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. The results regarding lipid peroxidation in animal tumors and plasma suggest the involvement of reactive oxygen species as the mechanism leading to the killing of tumor cells as a consequence of PDT.

Analyzing the differences between the group treated with Chiroxy and irradiated and the group treated with Base III and irradiated, results that topical Chiroxy and irradiation with red light produce higher levels of protein carbonyls in plasma and tumor (p=0.01).

Same increased levels of serum PC. (p=0.04) are seen in group treated with Chiroxy and irradiated in comparison with group treated with PDT 5-ALA 20% in Base III. The effects of Chiroxy on PC, as a unique substance applied on tumors in this study, are similar with those obtained on PDT 5-ALA 20% in Base III or Chiroxy. This aspect confirms the capacity of Chiroxy to generate protein carbonyl.

The serum level of lipid peroxidation markers shows that Chiroxy does not increase lipid oxidation, on the contrary it decreases it. The same tendency is found in tumor but has no statistical significance (p=0.53). These changes of oxidative stress markers prove the pro-oxidative effect on proteins and insignificant effects on lipids.

Adding Chiroxy to 5-ALA 20% increases the level of PC in tumor in comparison with those treated only with Base III and irradiated.

In our experimental model, PDT with Chiroxy added to 5-ALA 20% does not result in statistic different effects in comparison with simple PDT with 5-ALA 20% in Base III. This explains that 5-ALA included in Chiroxy has no additive photodynamic effects. Beside, the addition of Chiroxy to 5-ALA does not intensify protein oxidation in comparison with Chiroxy alone. The results of our study indicate that Chiroxy can be used in pre-treatment as an oxygen generator necessary for amplification of photodynamic effect before PDT. The association of Chiroxy and 5-ALA followed by irradiation doesn't offer better therapeutic results in increasing oxidative stress in PDT.

CONCLUSIONS

On carcinomas Walker treated with PDT in different combinations (5-ALA 20% in Base III, 5-ALA 20% in Chiroxy) we obtained an increased oxidative stress proved by high levels of protein carbonyls which are markers of reactive oxygen species of proteins.

Compared to Base III, topical Chiroxy combined with irradiation on Walker carcinomas generate oxidative stress.

This is a preliminary study of PDT that investigates the oxidative stress produced by Chiroxy and a photosensitizer and further work has to be done in this direction.

This study needs to be followed by other studies of PDT in which Chiroxy and photosensitizer should be applied on a skin tumor and oxidative stress can be investigated.

REFERENCES