EXPERIMENTAL STUDY OF THE NEUROTOXIC EFFECTS OF PHOTODYNAMIC THERAPY ON THE SPINAL CORD

ABSTRACT

Objective: To evaluate the effects of photodynamic therapy (PDT) on the dura mater using the photosensitizers aluminum chloride phthalocyanine and methylene blue in vivo assays. Methods: Fifty-six male Wistar rats were divided into two groups; one submitted to PDT and the other submitted to the photosensitizers without their photoactivation (control). The photosensitizers were applied to the dura mater after laminectomy at the T10 level. The methods used for assessment were the Basso, Beattie and Bresnahan (BBB) functional evaluation scale and study of the dura mater by light microscopy. Results: No changes in motor activity were observed in the animals submitted to PDT compared to control. Histological and pathological evaluation did not show any differences between the group exposed to activated photosensitizers and the control group with regard to the inflammatory process and tissue necrosis. Conclusion: The joint use of PDT with the photosensitizing pharmaceuticals aluminum chloride phthalocyanine and methylene blue did not induce any clinical neurotoxic effects or histological changes in the dura mater of the animals studied. 

Keywords: Photochemotherapy; Dura Mater; Methylene Blue; Laser.

RESUMO

Objetivo: Avaliar os efeitos da terapia fotodinâmica (PDT) na dura-máter usando os fotossensibilizadores cloreto de alumínio ftalocianina e azul de metileno em ensaios in vivo. Métodos: Cincuenta e seis ratos Wistar machos divididos em dois grupos; um submetido à PDT e o outro submetido aos fotossensibilizadores sem a fotoativação (controle). Os fotossensibilizadores foram aplicados sobre a dura-máter depois de laminectomia no nível T10. Os métodos de avaliação usados foram a escala de avaliação funcional de Basso, Beattie e Bresnahan (BBB) e o estudo da dura-máter por microscopia óptica. Resultados: Não foram observadas alterações na ativação motora dos animais submetidos à PDT com relação ao grupo controle. A avaliação histológica e histopatológica não mostrou diferenças entre o grupo exposto aos fotossensibilizadores ativados e o grupo controle, com relação ao processo inflamatório e à necrose tecidual. Conclusões: O uso conjunto de PDT e os fármacos fotossensibilizantes cloreto de alumínio ftalocianina e azul de metileno não induziu efeitos neurotóxicos clínicos e/ou alterações histológicas sobre a dura-máter dos animais estudados.

Descritores: Fotoquiomerapia; Dura-Máter; Azul de Metileno; Laser.

RESUMEN

Objetivo: Evaluar los efectos de la terapia fotodinámica (PDT) en la duramadre utilizando los fósforosensibilizadores de ftalocianina de aluminio clorado y azul de metileno en ensayos in vivo. Métodos: Cincuenta y seis ratos Wistar machos se dividieron en dos grupos; uno fue sometido a PDT y el otro submetido a fotosensibilizadores sin fotoactivación (control). Los fósforosensibilizadores se aplicaron a la duramadre después de la laminectomía en el nivel T10. Los métodos de evaluación utilizados fueron la escala de evaluación funcional de Basso, Beattie y Bresnahan (BBB) y el estudio de la duramadre mediante microscopía óptica. Resultados: No hubo cambios en la actividad motora de los animales sometidos a PDT en relación con el grupo de control. La evaluación histológica y histopatológica no mostró diferencias entre el grupo expuesto a fotosensibilizadores activados y el grupo de control con respecto al proceso inflamatorio y la necrosis tisular. Conclusiones: El uso conjunto de PDT con las sustancias fósforosensibilizadores ftalocianina de aluminio clorado y azul de metileno no indujo efectos neurotóxicos clínicos o cambios histológicos en la duramadre de los animales estudiados.

Descritores: Fotoquimioterapia; Duramadre; Azul de Metileno; Rayos láser.
INTRODUCTION

Photodynamic therapy (PDT) is a promising treatment for cancer and non-oncological diseases. It combines a photosensitizer drug (PS, organic dye) with visible light irradiation in the range of the electromagnetic spectrum of appropriate wavelength, and oxygen, each of which alone is harmless. The photodynamic effect produces reactive oxygen species (ROS), which function as lethal cytotoxic agents that inactivate tumor cells, bacteria and other living species. Interestingly, the photosensitizer drug is preferentially absorbed by the desired tissue, and the light irradiation is limited to a specific region. This confers dual selectivity to the PDT technique. Hence, PDT allows for the selective activation of target tissue while healthy tissue remains intact. The mechanism of cell death could be by classical necrosis or apoptosis. Hematoporphyrins were the first generation of PS applied in clinical PDT procedures. With the aim of improving PDT efficacy by increasing diseased tissue penetration, researchers have developed the second generation of PS drugs, phthalocyanine being the main representative of this generation.

The preclinical application of such drugs has seen a significant rise in many fields in the last decade.

Aluminum chloride phthalocyanine (AICIPc) is a chemically stable second-generation photosensitizer that displays adequate photophysical properties (such as high triplet quantum yields and long triplet lifetimes) for PDT. Methylene blue (MB), a generation of AICIPc that is also adsorbed in the visible range of the spectrum, also leads to a biological response in the tissues of different species. Even though AICIPc has high molecular weight, its hydrophobic nature facilitates its interaction with lipid bilayers. AICIPc-PDT has been successfully used in cancer models, affording a relevant safety margin in medical trials. The procedures described above could be used for topical or systemic application, with target specificity as desired.

The advantage of PDT over other clinical procedures is that it has no toxic side effects and it can be applied repeatedly, over a short period of time, with low risk to the patient. PDT has also been frequently used for photodiagnostic purposes, since most of the PS used have the ability to absorb light and to produce active species (ROS) or emit light as fluorescent probes for diagnosis.

Given that PDT has become a first choice treatment for many diseases, and in view of the absence of data showing the effect of PDT on the nervous system, the objective of this study was to analyze this behavior and to elect PDT for application as a therapeutic treatment for spinal diseases. It therefore assesses, experimentally, the neurotoxic effects of PDT and the photosensitizers AICIPc and MB on the spinal cord and dura mater of rats, investigating any changes in motor functions of the nervous system.

METHODS

The study was approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine of Ribeirão Preto - USP (protocol no. 005/2014). Fifty-six male Wistar rats weighing 250-300 g were divided into 2 experimental groups. In Group A (intervention), the animals were anesthetized with a mixture of 20 mg/ml xylazine (0.7 ml/100 g) and 50 mg/ml ketamine (1ml/100 g). Laminae were then sutured plane by plane, and the animals were kept in 20 x 40 cm polypropylene cages (type C), with 4 animals to a cage.

The slides of groups A and B were analyzed by counting pixels in the regions stained with hematoxylin-eosin. (Figure 1) Hematoxylin stains the cell nuclei, while eosin stains the extracellular matrix for the assessment of the integrity of nuclei and cells, respectively. Thus, we compared the integrity of the meninges and the neurons (number of nuclei and cytoplasms) and determined the presence or absence of inflammatory cells in the various groups by counting the pixels of each image in relation to the control group and to a standard healthy model.

Histopathological analysis was performed by a pathologist of the Pathology Group of the University Hospital, Faculty of Medicine of Ribeirão Preto, USP Ribeirão Preto (SP) Brazil, who determined the presence of dead cells due to necrosis or the production of inflammatory cells. The slides were examined under an Olympus BX40 light microscope with oil objectives at 400X.

The degree of inflammatory infiltrate was quantitated as follows: + (less than 20 inflammatory cells/400X magnification field); ++ (21 to 50 cells/400X magnification field); +++ (more than 50 cell/400X magnification field).

Statistical Analysis

The data were analyzed statistically by one-way analysis of variance (ANOVA) between the treatments performed in the subgroups. The Tukey post-test was applied, and the level of significance was set at 5% (p<0.05). The analysis was carried out using the software program Axiomvision LE V.4.8.2.0 (Zeiss).
Table 1. Results of the Basso, Beattie and Bresnahan (BBB) scale for locomotor assessment applied to the animals under study.

| Score | Characteristics | Comments |
|-------|-----------------|----------|
| 0     | No hindlimb (HL) movements are observed | Mild – Less than 50% of joint capacity |
| 1     | Mild movements of 1 or 2 HL joints | Extensive – More than or equal to 50% of joint capacity |
| 2     | Extensive movement of 1 joint and possible mild movement of another HL joint | Two joints = generally, hip and knee |
| 3     | Extensive movement of 2 HL joints | Three joints = hip, knee, and ankle |
| 4     | Mild movements of all 3 joints | 3rd joint = ankle |
| 5     | Mild movement of 2 joints and extensive movement of the 3rd HL joint | |
| 6     | Extensive movements of 2 joints and delicate movement of the 3rd HL joint | |
| 7     | Extensive movements of the 3 joints | |
| 8     | Mild movements without supporting body weight or paw resting without supporting body weight | Rhythmic extension of 3 HL joints, with the trunk positioned sideways |
| 9     | Plantar rest with support of immobile body weight or occasional, frequent or consistent support of body weight with dorsal rest | Weight support = muscle contraction. HL extension during plantar rest of the paw or elevation of the immobile pelvis |
| 10    | Step occasionally supporting body weight, with no coordination between forelimbs (FL) and HL | Occasionally > 5% and lower than or equal to 50%. Steps – plantar contact with weight support; the HL advances to reestablish plantar contact. Coordination – simultaneous movements between the HL and FL, alternating between sides |
| 11    | Frequent to consistent steps with weight support without coordination between HL and FL | Frequency – 51 to 94% of the time; Consistent – 95 to 100% of the time |
| 12    | Frequent to consistent steps with support of body weight and occasional coordination between the FL and HL | 60 to 50% coordinated locomotion |
| 13    | Frequent to consistent steps with support of body weight and frequent coordination between FL and HL | 51 to 95% coordinated locomotion |
| 14    | Consistent step coordination with plantar rest and predominant position of the paw and rotation in the initial contact and in elevation, frequent plantar steps, consistent coordination between FL and occasionally with dorsal rest | Rotation => internal and external rotation of the hind paw when it is resting and raised |
| 15    | Consistent step coordination with plantar rest, predominant position of the paw in parallel in the initial contact and in its elevation | Frequent liberty of the 1st toe => More than half the steps occur with no scratching sounds |
| 16    | Consistent step coordination with plantar rest, predominant position of the paw in parallel in the initial contact and in its elevation | |
| 17    | Consistent coordination of the step with plantar rest and consistent liberation of the 1st toe. Position of the paw in parallel in the initial contact and in its elevation | Consistent scratching with 4 toes for a period of 4 minutes |
| 18    | Consistent coordination of the step with plantar rest, consistent liberation of the 1st toe. Position of the paw in parallel in the initial contact and in its elevation, with the tail downward most of the time | Tail downward => tail touches the floor during the steps |
| 19    | Consistent coordination of the step with plantar rest, consistent liberation of the 1st toe. Parallel position of the paw in the initial contact and in elevation, the tail consistently upward and trunk instability | Tail elevated => does not touch the floor. Trunk instability => trunk lateralization when turning rapidly (loss of balance) |
| 20    | Consistent step coordination with plantar rest, consistent liberation of the 1st toe. Parallel position of the paw in the initial contact and in elevation, the tail consistently upward and trunk instability | |
| 21    | Consistent coordination during walking, consistent movement of the 1st toe. Parallel position of the paw during rest and elevation. Tail upward. | Consistent trunk stability, without wobbling or falling, the movement of the pelvis and tail coordinated with locomotion. |

Figure 1. Histological sections stained with Hematoxylin (A) and Eosin (B) after incubation of different samples: PS = physiological solution; MB = methylene blue; AICIPc = aluminum chloride phthalocyanine; MB+AICIPc = methylene blue + aluminum chloride phthalocyanine.
RESULTS

The effects of PDT on the dura mater after T10 laminectomy were analyzed according to the following criteria: a) neurological signs and symptoms, b) quantity of necrotic tissue, and c) quantity of inflammatory cells, comparing the experimental group to the control group. Thus, it was possible to observe the effect of visible laser at 660 nm on the photoactive agents studied in their specific formulations, which guaranteed their best interaction in an isolated and joint manner when applied to the dura mater with and without photoactivation.

All animals were examined. None of them showed neurological deficits. All of them reached 21 points on the BBB scale after recovery from anesthesia and on the seventh postoperative day (168 hours), as shown in Table 1.

The slides of groups A and B were analyzed by pixel count after staining with hematoxylin and eosin (Figure 1), assessing and comparing the integrity of cell nuclei and cytoplasm between the experimental and control groups. Thus, it was possible to assess the integrity of the dura mater, as well as the behavior of the neurons in the various groups.

Group A was divided into subgroups treated with saline solution, AICIPc, MB and MB + AICIPc, plus treatment with visible laser at 660 nm frequency. This enabled us to analyze the individual neurotoxicity of each substance, as well as the combined action of both after laser treatment, and to compare the results to those of the control group (saline solution + laser). As anticipated, no significant difference (p < 0.005) was observed between groups, as shown in Figures 2 and 3.

Group B (with no light treatment) was also divided into subgroups as Group A. The individual and combined neurotoxicity of the substances were analyzed without the influence of laser and compared to the control (saline solution). No significant difference (p < 0.005) was detected, as shown in Figures 4 and 5.

Histopathological analysis did not reveal tissue necrosis, and an inflammatory infiltrate with the presence of lymphocytes was detected on the following slides:

DISCUSSION

The functional, histological, pixel count and histopathological analyses and quantification of the processes for hematoxylin and eosin staining showed that PDT with the photosensitizer pharmaceuticals (PPs) in question (AICIPc and MB) had no neurotoxic effects when applied directly to the dura mater. They also showed no motor changes, cell death or apoptosis, or significant inflammatory activity. It should be pointed out that both agents used in our study have a tissue is differentiated.

Data on the clinical applications of PDT are limited within this biological context and most of the previous studies using PDT have assessed its proven antineoplastic and antimicrobial actions. Also, some clinical antibacterial studies have been reported. Numerous clinical trials were reported in the 1970s for the treatment of herpes simplex with photoactivation in recurrent lesions. A 1% neutral red solution was applied topically, followed by exposure to a 40 W incandescent tungsten lamp for 15 minutes. The treatments, however, were interrupted due to the low efficacy and...
possible carcinogenicity of the method. Indeed, it was not known, at that time, that it would be necessary to use monochromatic lights to ensure greater absorption by the phototoxic agents, or that these lights would be able to activate the photochemical and photobiological pathways of the cell response, with complete safety and avoiding side effects. In recent studies, PDT using photosensitizers such as 5-aminolevulinic acid (ALA), hematoporphyrin derivatives (HPD, Photofrin), talaporfin sodium, or meta-tetra(hydroxyphenyl)chlorin (mTHPC) has proven to be a positive adjunctive treatment for skin cancer and brain tumors in the clinical setting. Therefore, the theranostic properties of PDT could improve control of the local action of reactive species produced during the classical treatment.\(^{1,2,5,25}\) Most of the work done with 5-ALA has been conducted in clinical trials in many countries around the world, particularly Brazil, in studies conducted at the University of Sao Paulo, Ribeirão Preto, focusing on neoplastic diseases, with excellent results. ALA has also proved to be efficient for the treatment of molluscum contagiosum (caused by poxvirus, HPV), the virus that causes vesicular stomatitis.\(^{27}\) Abdel-Hady et al.\(^{28}\) used topical ALA-PDT to treat neoplastic vulvar lesions, but observed a short-term response in only one third of cases. The authors consider AICIPc to be a stable second generation photosensitizer drug with optimal wavelengths in most of the electromagnetic spectrum (610-660 nm).\(^{12}\) This photosensitizer has previously induced apoptotic cell death in glioma cells.\(^{29}\) AICIPc is a chemically stable second-generation photosensitizer that displays adequate photophysical properties (such as high triplet quantum yields and long triplet lifetimes) for PDT.\(^{12}\) Even though AICIPc has high molecular weight, its hydrophobic nature facilitates its interaction with lipoid bilayers. AICIPc-PDT has been successfully used in cancer models, affording a relevant safety margin in medical trials.\(^{13,15}\) The absorption and emission of light at long wavelength PS and the nano-drug delivery system enable deeper lesions to be reached.\(^{31,32}\)

The chemical structure contains a variety of metal ions which are responsible for the particular properties of each molecule. Aluminum (III) and zinc (II) are two metal ions used to improve phototoxicity. Its application to the treatment of extradural disease could be a highly useful tool and a field to be explored. The limitations of the present study were the absence of comparative studies in the literature, the large number of photosensitizers and laser types with similar characteristics, and the small number of animals used. Immunohistochemistry and electron microscopy studies will be necessary for more detailed experiments in order to obtain a more in-depth analysis of the interaction of PDT and photosensitizers on the dura mater and neural tissue.

**CONCLUSION**

Neurological and histopathological assessment showed that PDT with AICIPc and MB does not cause neurotoxic changes. The application of laser and photosensitizers did not induce functional changes in the meninges or neurons. PDT has been used for the treatment of spinal lesions with AICIPc excited by infrared light, reducing the density of glial cells in the epicenter of the lesion, and thus inhibiting the formation of a glial scar and reducing the chance of neurological deficit.\(^{35}\)

In the present study, histological analysis by pixel count and histopathological analysis of the slides revealed no significant structural damage to the dura mater or the neurons. The pixel count showed that the number of cell nuclei and cells (intact cytoplasm membranes) was similar in the subgroups when the photosensitizers were applied separately or in combination (Figures 2, 3, 4 and 5) and no cell death or apoptosis was observed. Microscopic examination did not reveal significant inflammatory activity. Only a small local inflammatory lymphocyte infiltrate was detected on 4 slides, as described in the histopathological analysis section. Lambert et al.\(^{36}\) reported that PDT using cationic porphin inactivated Candida albicans by damaging the cytoplasm membrane. Electron microscopy images clearly showed that the damage produced occurred from the outer to the inner layers of the cytoplasm membrane, and confocal fluorescence images showed that a massive afflux occurred after cell death.\(^{36}\) The reduction of the number of bacteria mediated by PDT in a burn wound was rapid (less than one hour) compared to standard therapy (silver sulfadiazine cream) which required several days to complete treatment. Cell segmentation by the PPs prevented recolonization and had a protective effect on the tissues.\(^{37}\)

The present use of PDT with AICIPc and MB was found to be safe for the dura mater and did not cause significant neurotoxic changes. Its application to the treatment of extradural disease could be a highly useful tool and a field to be explored. The limitations of the present study were the absence of comparative studies in the literature, the large number of photosensitizers and laser types with similar characteristics, and the small number of animals used. Immunohistochemistry and electron microscopy studies will be necessary for more detailed experiments in order to obtain a more in-depth analysis of the interaction of PDT and photosensitizers on the dura mater and neural tissue.

CONCLUSION

Neurological and histopathological assessment showed that PDT with AICIPc and MB does not cause neurotoxic changes when applied to the dura mater of Wistar rats. Based on these observations, we conclude that PDT, AICIPc and MB offer excellent perspectives for use in further clinical trials.

All authors declare no potential conflict of interest related to this article.

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