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Histochemical study of the response mechanisms of the nasal polyp tissue associated with the laser interstitial thermotherapy

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ABSTRACT

The paper presents the results of the histochemical study of the polyp tissue morphofunctional state associated with the laser interstitial (1 to 10 W) thermotherapy. It suggested the laser induces apoptosis of the polyp tissue cells via mitochondrial pathway, which is supported by the positive staining for caspase 3 and is dose-dependent. The study demonstrates that the inhibition of apoptosis by Bcl-xL occurs at the low laser intensity (1-5 W) and further (5-10 W) the process becomes unidirectional.

Keywords: chronic polypoid rhinosinusitis (CPR), histochemistry, caspase 3, Bcl-xL, interstitial laser thermotherapy

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INTRODUCTION

Despite the large number of scientific papers on the etiopathogenesis, clinical picture, and modern methods of diagnosis and treatment of polypoid and other hyperplastic processes in the nasal cavity, there are relatively few papers dealing with the morphology of these pathological response mechanisms [1-5].

We attempted to elucidate the response mechanism of the nasal polyp tissue during treatment with laser radiation (1-10 W) via histochemical studies identifying the levels of markers of apoptosis (caspase 3, Bcl-2) and satb2.

MATERIALS AND METHODS

The morphological examination of nasal polyps was performed in 15 patients with chronic polyp rhinosinusitis (CPR). The tissues of the excised polyps (N=15) fixed in 10% neutral formalin "Histoline" ("Element Limited", Sankt-Petersburg, Russia) were sectionized and stained with hematoxylin and eosin ("Reactiv Plus Limited", Arkhangelsk, Russia). The material was analyzed using the microscope Axio Observer A1 (Zeiss, Berlin, Germany) with a digital camera AxioCam and software for morphometric studies AxioVisionRel. 4.8. Statistical processing of the obtained values was performed via Microsoft Excel 2007.

The immunohistochemical studies were performed using the 18 µm polyp tissue cryosections according to standard methods [6]. The sections were washed in 1xPBS with 0.2% TritonX-100 used as a detergent. Antigen retrieval was performed in a hot 10 mM citrate buffer with pH=6 for 10 minutes. In experiments to localize satb2, endogenous peroxidase was blocked by incubating the sections in the 1.5% hydrogen peroxide in methanol for 15 min. Nonspecific binding was blocked by incubation in the 1.5% BSA solution in 1xPBS with 0.2% Triton for 2 hours at room temperature. The sections were then incubated with primary antibodies (1:200) diluted in 1.5% BSA in 1xPBS with 0.2% Triton overnight at +4°C. In our study, we used the following primary antibodies to proteins: SATB2 (sc-8137, Santa Cruz), Bcl-xL (sc-8392, Santa Cruz), Cleaved Caspase-3 (№9661, Cell Signaling).

For the immunofluorescent identification of active caspase and Bcl-xL, the next day the sections were washed in 1xPBS and incubated with the corresponding fluorescently-labeled secondary antibodies (Alexa Fluor 488 Donkey Anti-Rabbit IgG Antibody and Alexa Fluor 555 Donkey Anti-Mouse IgG Antibody) (1:400) for 2 hours at room temperature. The sections were then washed in 1xPBS and put beneath the cover glass in MOWIOL containing DAPI as a nuclear stain.

For satb2 immunoperoxidase staining, after the incubation in primary antibodies, the sections were also washed and incubated with the corresponding antibodies labeled with horseradish peroxidase (Horseradish Peroxidase-Labeled Donkey Anti-Mouse IgG Antibody) for 2 hours at room temperature. The sections were then washed in 1xPBS and the peroxidase activity was detected using a 3,3'-diaminobenzidine (5 mg/ml) solution containing 0.03% hydrogen peroxide in 0.2M tris buffer (pH 7.54).

To identify possible ways of cell death, we used the method of immunohistochemical determination of both apoptosis markers - active caspase 3, the main executive apoptotic protease, and anti-apoptotic protein Bcl-xl [7; 8]. We also evaluated the tumor marker satb2 that is a transcription factor [6; 9]. Satb2 (special AT-rich sequence-binding protein 2) is involved in the creation of a specific chromatin structure establishing a platform for binding the rest of transcription factors [10; 11], so it can be used as a criterion for the state of chromatin.

RESULTS

Previously [12], we showed that the laser interstitial (1 to 10 W) thermotherapy had no influence on the structure and physiology of epithelial glands. The therapeutic effect is achieved due to the effects of radiation on the loose connective tissue, which results in the migration of lymphocytes and eosinophils to the focus of inflammation, changes in its structure (coarsening and thickening of connective tissue fibers) and, finally, reduction in the volume of the enlarged polyp tissue.

In particular, in groups, where laser radiation of 8 to 10 W was used, we detected the expanding areas of coagulation, as well as growing randomness and thickening of connective tissue fibers (Fig. 1).

As seen in Fig. 1, laser radiation causes significant destructive changes in the loose connective tissue and we need to find out the mechanism of this process – either apoptosis or necrosis. To identify a particular mechanism of various-intensity laser radiation influence, we used the method of immunohistochemistry.

When stained with DAPI, no experimental group showed the differences in the amount of fragmented nuclei. Also, there was no positive response to satb2 (Fig. 2).

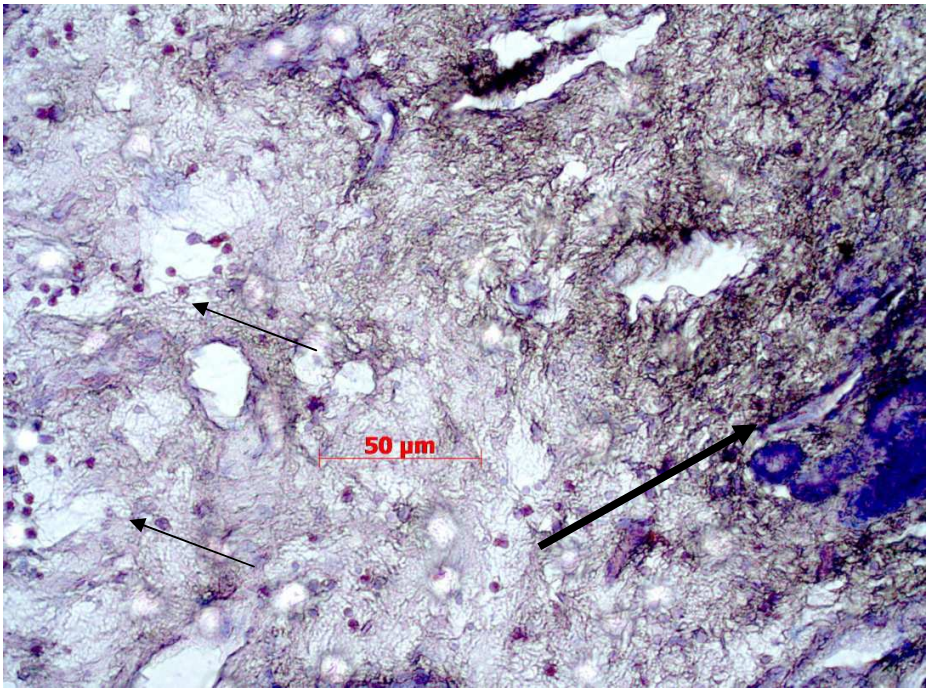


Fig. 1. Polyp tissue in the group, where laser radiation of 9 W was used. In the polyp stroma, you may see a laser exposure area, in which coarse fibers of connective tissue and area of necrotic changes predominate (indicated by a large arrow). In the area of the minimum laser exposure, you may see eosinophilic infiltration (indicated by small arrows). Hematoxylin and eosin. Magnitude x 200

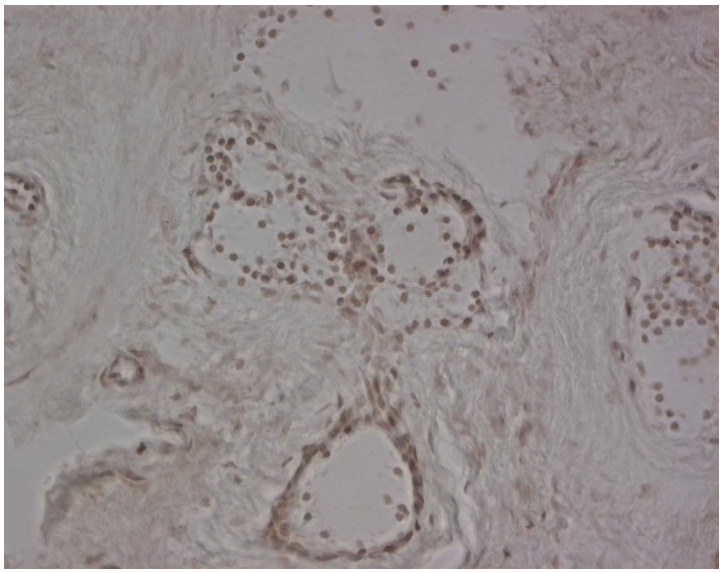
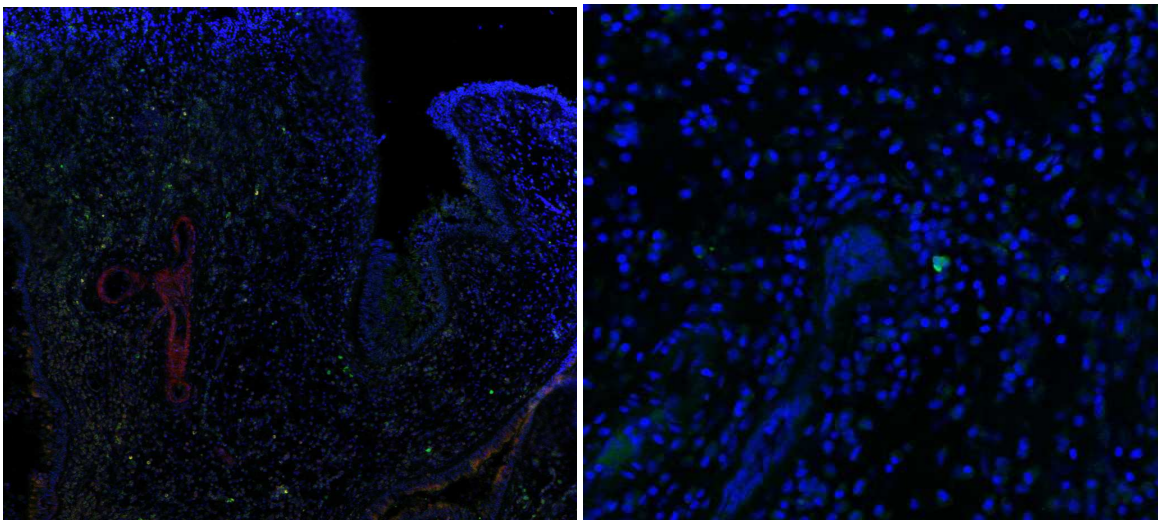


Fig. 2. Polyp tissue in the group, where laser radiation of 1 W was used. You may see a negative response to satb2. Magnitude x 200

The split activated form of caspase 3 is a key executive apoptotic protease and therefore it was chosen for the detection of apoptosis [13; 14]. In contrast, Bcl-xL is involved in the regulation of apoptosis and suppresses it [15; 16], that is why this protein is considered one of the key elements in the survival of cells.

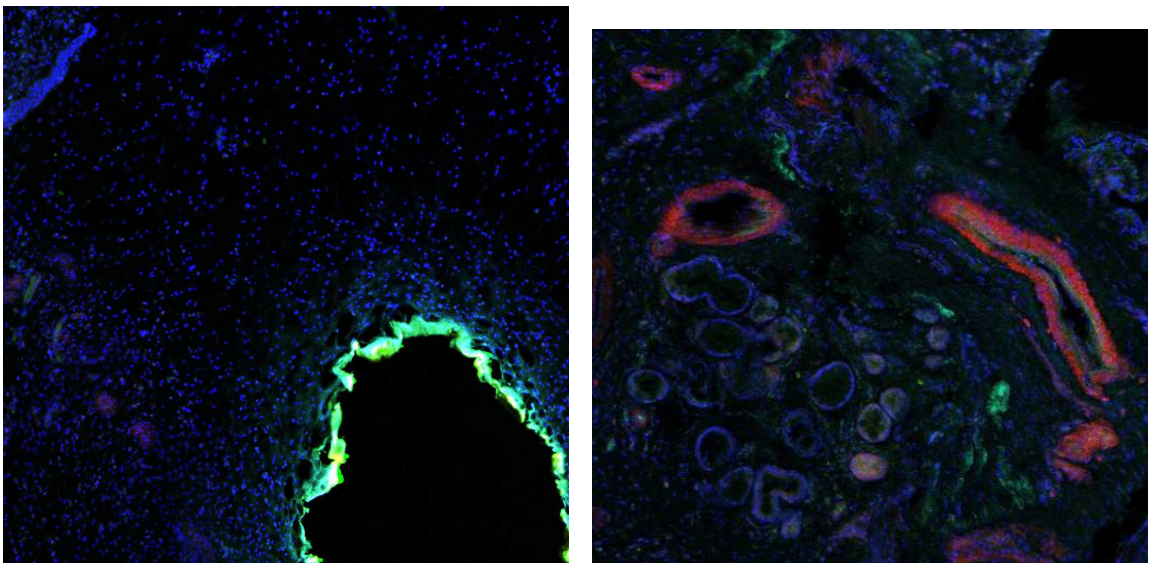
Due to positive staining for the activated caspase 3, apoptosis was detected in groups with different laser radiation intensity (1-10 W) (Fig. 3).



A B

Fig. 3. Polyp tissue in the group, where laser radiation of 1 W (A) and 10 W (B) was used. There is a positive response to apoptosis (you may see caspase 3 as green dots) and Bcl-xL, which shows the synthetic activity of the glands (pink color). Bcl-xL staining and active caspase 3. Magnitude: A) x 100, B) x 200

Also in groups, where a low laser dose was used (1-5 W), we found Bcl-xL positive staining (Fig. 4).



A B

Fig. 4. Polyp tissue in the group, where laser radiation of 1 W (A) and 5 W (B) was used. A) There is a positive response to caspase 3 (green dots and the area in the middle) and Bcl-xL, which shows the synthetic activity of the glands (pink color). B) You may see a laser coagulation area with strongly positive staining for caspase 3. Bcl-xL staining and caspase 3. Magnitude: A) and B) x 100

Fig. 5B shows a laser coagulation area that has a strongly positive reaction to caspase 3, which is explained by the activation of apoptosis rather than necrosis due to the low radiation intensity (only 5 W).

Also the use of laser of 5-10 W showed no positive staining for Bcl-xL.

DISCUSSION

The results obtained indicate the following. First, the laser radiation in these doses causes apoptosis in the loose connective polyp tissues. Most likely, apoptosis is activated via caspase 3. The activation of apoptosis by laser radiation has long been known [17], but do not forget that caspases can be activated in proliferating lymphocytes as well [18]. The version that apoptosis is carried out via caspase 3 is supported by the fact that the number of cells positively stained for caspase 3 grows as the intensity of the laser radiation increases, whereas the activation of lymphocytes by laser is not dose-dependent [19].

Also note that when the low-intensity laser radiation is applied you may observe the control of apoptosis by the inhibition via Bcl-xl. However, when the dose is significant (5-10 W), the process becomes unidirectional.

As a working hypothesis, which explains the morphology of changes in the polyp tissue due to an increase in the radiation dose, we may suggest that the first effector component of this mechanism are laser-activated fibroblasts that produce cytokines and modulate the structure of the connective tissue by producing mucopolysaccharides. Cytokines produced by the activated fibroblasts attract eosinophils and lymphocytes to the focus of inflammation, which is confirmed by our observations [12]. Eosinophils, in turn, increase vascular permeability and vasodilatation, which further positively affects the regeneration process.

As shown by our results, most likely, the polyp tissue cell death under laser radiation occurs through the mitochondrial pathway. This process is dose-dependent and evidenced by positive staining for caspase 3.

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