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# Expression of Nitric Oxide Synthase in Inflamed Gingival Tissue: Immunohistochemical and Image Analysis

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**Abstract:** We have used 12 Epulis taken during surgical operation and as control, some normal gingival specimen. The aim was to clarify the relationship between the degree of inflammation and NO production in several cases of Epulis, exhibiting different degree of inflammatory process and verifying the presence of cells involved in NO production. From the immuhistochemical data in our possession we can conclude that NO is one of the factor which module the pathogenecity degree of Epulis.

**Key words:** Epulis, NO, Immunoistochemistry, synthases, tissue

## INTRODUCTION

Physiological, pharmacological and histochemical studies indicate that Nitric Oxide (NO) is a novel and unusual gaseous (Rastoln, 1997) signalling molecule in several cell types (Moncada *et al*., 1991; Bredt and Snyder, 1992).

NO formation is catalyzed by a family of NO synthases (nNOS, eNOS, iNOS) that use L-arginine as substrate (Moncada and Higgs, 1993) . It is known that in the periodontal diseases iNOS is increased in neutrophils cells (Batista *et al*., 2002). It has been also suggested that neutrophils cells represent an additional activation pathway of the periodontal disease.

Epulis is a fibrous connective lesion that common occur in oral mucosa secondary to inflammation or injury, to congenital reason or proliferation of endothelial cells. This type of lesion is considerate as a chronic process in which there is overexuberant repair (granulation tissue) following, occasionally, a single causative event such as trauma or following continued low-grade injury (Leone *et al*., 2002). The lesion presents a submucosal masses that may become ulcerated secondarily to trauma. The colour can be light because of a relative increase of collagen or red because of an abuntance of well vascularized granulation tissue. There is not proliferation of nerves with the reactive hyperplastic tissue; this is the reason why Epulis are painless. Epulis rarely affects bone.

Although there are all pathogenically related lesions, different names or subdivision have been devised because of variation in anatomic site, clinical appearance, or microscopic picture.

In our findings we considered the morphological caracterization of the point of view strictly histological.

The aim of this study is to clarify the relashionship between the degree of inflammation and NO production in several cases of epulis exhibiting different degree of inflammatory process and verifying the presence of cells involved in NO production.

## MATERIALS AND METHODS

We have used 12 epulis taken during surgical operation from the files of the department of stomatology, Dental School, in Palermo. We have used also a normal gingival specimen taken during dental extraction as negative control.The histopathological diagnosis was made in Institute of Anatomy Pathology, Medical School in Palermo. All the specimens were fixed in Bouin's mixture and embedded in paraffin. Samples were stained with Hematoxilin and Eosine for the morphological exam. We investigated the immunohistochemical distribution of inducible NOS (iNOS). Sections with 5$\mu$m of thickness were collected on microscope slides 76×26 mm (Objektträger, Menzel-Glaser, Germany).The immunohistochemical staining was made using a monoclonal antibody against iNOS (Transduction laboratories-Lexington KY,USA) purified rabbit anti-iNOS type II Pab, 150 μg, 0.6 mL 250 μg $mL^{-1}$. The sections were processed for immunohistochemistry by the

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standard streptavidin-biotin complex method kit ultraystain polyvalent strept ABCHRP (Ylem s.r.l.- Roma-Italy). After incubation period with antibody (1:200) diluted in PBS with BSA 0,1% sections were washed with PBS and Igepal. Sections were washed again in PBS and incubated with polyvalent antiserum prediluted for 10 minutes, one more washing with PBS and incubation with streptavidin POD for 20 min.

The revelation of the immunocomplex was obtained with 3-Amino-9 Ethylcarbazole(AEC). Following immunostaining the sections were covered with water-soluble mounting medium (DAKO paramount aqueous mounting medium) and observed with Leica microscope.

Subsequently all the specimens were studied with Image Analysis Nikon-Lucia M system to obtain colorimetric data.

**Image analysis:** The image analyses are affected with the image analysis software Lucia M by Nikon, all the sections are observed with the Nikon microscope. The number of positive cells and the total number of inflammatory cells were enumerated per 15 consecutive microscopic fields (×400) (each field has area equal to 0,00025316 pixel ×pixel) in a representative section of each specimen. Results were expressed as mean±standard deviation (s.d.) 6 of *n* observations, × pixel2. The relative proportions of immunoreactive cells in each group were determined using the threshold method by a 30 point in the positive fields. All the results furnishing a colorimetric data related at the intensity of the reaction. These data are obtained with the mean saturation of the colour expressed as mean ± standard deviation (s.d.) of saturation divided the *n* of observation. (Image analysis was not made on Papillar Leukoplakia becouse inflammatory cells distribution, in this type of epulis, is different).

## RESULTS

The iNOS reactivity in the normal gingival tissue was nil. The samples of various histological types of epulis present an intense reactivity of iNOS so localized:

- *Fibro-angiomatosis epulis* is histological caracterized by the presence of very huge amount of fibres structures, plasma cells and many vessels.

Plasma cells immunostaining of iNOS is cytosolic (Fig.1 and 2)

- *Papillar leukoplakia* is histologicaly caracterized by very important infiltration of inflammatory cells, especially in dermic papillas. Plasma cells, neutrophils and epithelial cells of the basal layer are positive (Fig. 3).

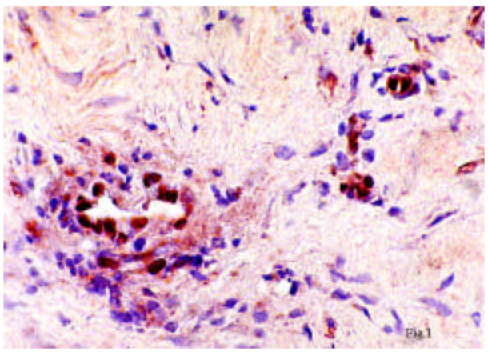

Fig. 1: Fibro-angiomatosis epulis-Fibers and few plasmacytes iNOS immunoreactive (hematoxylin counterstain-40X)

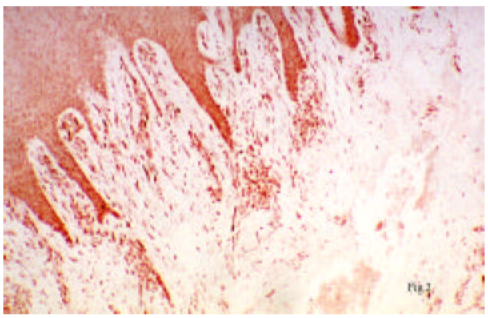

Fig. 2: Fibro-angiomatosis epulis-Fibers and few plasmacytes iNOS immunoreactive (hematoxylin counterstain-10X)

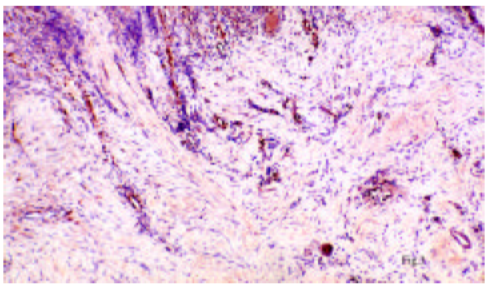

Fig. 3: Papillar leukoplakia - iNOS immunoreactivity in the inflammatory infiltrate located in the dermic papillas (20X)

- *Plasmacitary epulis* is histologicaly caracterized of a massive stroma invasion of plasma cells which are strongly immunostained (Fig.4), in this type of epulis endothelium is iNOS positive too (Fig. 5).
- *Giant cells epulis* is histologicaly characterized of giant cells with several nucleus, obtained from cells fusion and a considerable amount of inflammatory cells, all those structures are iNOS positive (Fig. 6).

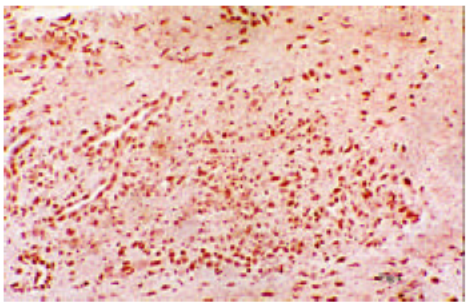

Fig. 4: Plasmacytary epulis-iNOS immunoreactivity in the inflammatory infiltrate (20X)

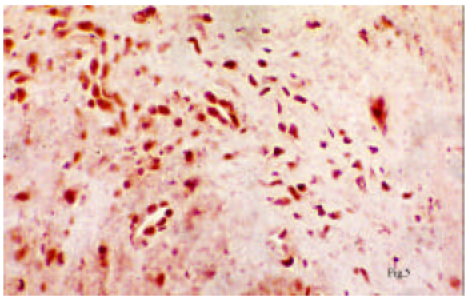

Fig. 5: Plasmacytary epulis-iNOS immunoreactivity in the inflammatory infiltrate (40X)

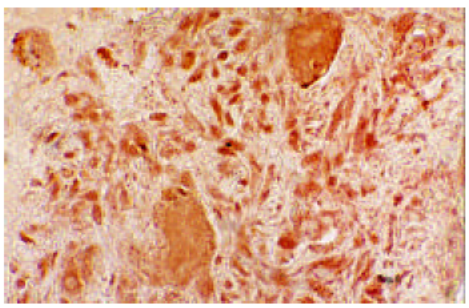

Fig. 6: Giant cells epulis-iNOS immunoreactivity in the giant cells (40X)

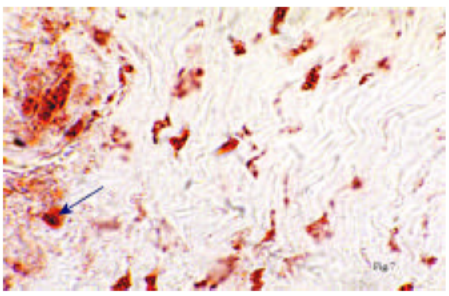

Fig. 7: Papillomatous epulis-iNOS immunoreactivity in the fibroblasts (40X)

Table 1: All the colorimetric data are obtained using 30 points of threshold in 40x microscopic area

| Media of colorimetric data | | | |
|---|---|---|---|
| Greater presence of plasma cells | | Greater presence of fibers | |
| ISTO 4 | ISTO 5 | ISTO 1 | ISTO 10 |
| 42.51±4 | 36.81±4 | 30.87±8 | 37.55±10 |

1.ISTO 1: Fibro-angiomatosis epulis, 2. ISTO 4: Plasmacytary epulis
3. ISTO 5: Giant cells epulis, 4. ISTO 10: Papillomatous epulis

- *Papillomatous epulis* is histologicaly caracterized by the presence of positive fibroblasts and particularly positive immunostaining in plasma cells is stronger.

These results are supported by the colorimetric data obtained with the image analysis method Lucia M by Nikon (Table 1).

## CONCLUSION

According to AC Batista's research (2002) the immunohistochemical analysis of the periodontal diseases shows a meaningful increase of iNOS cells positivity, regardless of the gingivitis or periodontopathy, particularly the positivity in cell where tissues present inflammation infiltration of neutrophils is strong.

In our research we to want evaluate the NO presence in several cases of Epulis, a particular lesion of the gingival mucosa. The results show, in all cases, iNOS positivity.

We think to be able to give a plausible explanation to our finding just for two cases, fibroangiomatosis epulis and Papillar Leukoplakia, after we compared them with literature data. For the other cases our finding remain, at present, a histotopochemical study, as in our previous publication (Leone *et al*., 2002). In the samples rich in collagen the iNOS positivity is limited to few cells around the vessels, in accordance with literature data which show that the presence of NO reduces the collagen synthesis (Okamoto *et al*., 1997). It is known that lynphociyte B cells and plasma cells have a more important role in the progression of chronic inflammation with respect of lynphocytes T cells (Seymour, 1987). These data are in accordance with our finding where the iNOS reactivity of the Papillar Leukoplakia is high in the epithelium and plasma cells. In relation to the high malignant transformation capacity of this type of epulis, we believe that this is probably due to the presence of NO (Seymour, 1987).

From the immunohistochemical data in our possession we can conclude that NO is one of the factor which module the pathogenicity degree of the epulis.

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