

# Effects of N-acetyl-cysteine and acetylsalicylic acid on the tonsil bacterial biofilm tissues by light and electron microscopy

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**Abstract. – OBJECTIVE:** The present study aimed to investigate the effects of the bacterial biofilm formation on the tonsil surface exposed N-acetyl-cysteine (NAC) and acetylsalicylic acid (ASA) of patients undergoing tonsillectomy by light and electron microscopy. The general process of biofilm formation comprises adhesion of free-living or planktonic bacteria to a surface, which subsequently develop into micro-colonies and form a biofilm. Based on studies that have shown the presence of biofilms in common sites of chronic infections, it has become clear that bacteria may persist on mucosal surfaces through formation of biofilms.

**PATIENTS AND METHODS:** Ten patients between 4 and 39 years of age (mean, 11.9 ± 11.2 years). In all cases, periodic acid Schiff (PAS) staining was found to be an accurate predictor of the presence or absence of biofilm using light microscopy as a control standard. Therapeutic doses of NAC and ASA were identified as the effective on the tonsil bacterial biofilm using light and electron microscopy.

**RESULTS:** Biofilm formation was detected on all samples. Tonsils removed from patients with ASA-10 had showed higher-grade inhibitory effect at the biofilm formation than the other group ( $p \leq 0.0001$ ). The correlation was found between drug dose and decrease at the biofilm formation.

**CONCLUSIONS:** In chronic or recurrent tonsillitis patients, decrease on the tonsils surface biofilm formation may be associated with ASA dose. Whether effect on the tonsils surface biofilm formation of other agent have a role is not known.

*Key Words:*

Acetylsalicylic acid, Chronic tonsillitis, *In vitro*, Mucosal biofilm, N-Acetyl-cysteine.

## Introduction

In spite of improving health standards and widespread use of antibiotics which reduce tonsil tissue infections, acute tonsillitis is a common disease on upper airways of childhood and adults with recurrences<sup>1</sup>.

The oxidative stress, the fibrin and the very large communication network play a role on bacterial biofilms formation. Mucosal biofilms are associated in tonsils removed for chronic or recurrent tonsillitis which have the adaptability to continue environmental conditions and they make negative results on host immune resistance. They also have an important role in chronic infectious diseases by composing of tissue damage through hydrolytic enzymes. However, they are play a relevant role in persistent infections such as chronic sinusitis and chronic otitis, as well as chronic bronchitis and bronchial colonization in patients with cystic fibrosis (CF)<sup>2-3</sup>.

Despite this, biofilm has been difficult to identify and study. Bacteria in biofilms have numerous defense mechanisms and, therefore, their response to antibiotics is usually incomplete<sup>4</sup>.

NAC (N-Acetyl-Cysteine) is an antioxidant agent which decreases the variety of bacteria on biofilm formation<sup>5</sup> and reduces the production of extracellular polysaccharide matrix<sup>6</sup> while promoting the disruption of mature biofilms<sup>5</sup>.

Previous studies showed that NAC reduced *in vitro* adhesion of *Streptococcus pneumoniae* and *Haemophilus influenzae* to human oropharyngeal epithelial cells<sup>6</sup> with elevated prostaglandin levels

in some chronic infections<sup>7</sup>. Similarly, cyclooxygenase (COX) inhibitors such as aspirine has also been shown to decrease the biofilm production and completely block hyphal formation in fungal infections<sup>8</sup>. The present study aimed to investigate the effects of NAC and ASA at the mucosal biofilms in humans by light electron microscopy in tonsils removed for chronic or recurrent tonsillitis.

## Patients and Methods

### *Application of Fixative and Drugs*

Total of 10 patients who tonsils removed for chronic or recurrent tonsillitis were used in the present study. The indications for tonsil removal were a recurrent tonsillitis in 3 (30%) and chronic tonsillitis in 7 (70%) patients. Tonsil specimens were cut into five equal pieces and were fixed in 10% neutral buffered formalin that were washed in phosphate-buffered saline. Five ml of NAC 4% (Muconex 200 mg/5 ml) syrup was dissolved in 15 ml saline to make a solution of % 1 NAC and a solution of ASA was prepared with one tablet (Aspirin tablet 100 mg) dissolved in 10 ml saline for each tissue. Tonsil specimens, control group expect, exposed 5 minute to NAC (Muconex<sup>®</sup>), 10 minute to NAC (Muconex<sup>®</sup>), 5 minute to ASA (Aspirin<sup>®</sup>) and 10 minute to ASA (Aspirin<sup>®</sup>) respectively.

Five horizontal equal sections from 10 tonsils were studied for a total of 50 samples. The surface epithelium of 1 tonsil was not intact, and it was omitted from further evaluation.

### **Histopathological Examination**

#### *Light Microscopy*

Specimens obtained from the tonsils were fixed in 10% neutral buffered formalin. After 24 h of fixation, the specimens were dehydrated in a graded alcohol series. After dehydration, the specimens were cleared in xylene and embedded in paraffin wax. Serial sections of 4-5  $\mu$ m thickness were obtained and stained with periodic acid-Schiff (PAS) to examine the thickness of the mucosal biofilm. The slides were examined under a light microscope (BH-2; Olympus, Tokyo, Japan), and photomicrographs were taken.

#### *Electron Microscopy*

The tonsil was removed from for electron microscopic examination, while the pieces of tonsil

tissues were fixed in 2.5% gluteraldehyde in 0.1 M sodium phosphate buffer. Following this, the tissue was fixed for 2 h in the same fixative at 4°C. The tissue was then thoroughly washed three times in a 0.1 M sodium phosphate buffer and post-fixed in 1% osmium tetroxide in a 0.1 M sodium phosphate buffer at 4°C. After repeated washing, the tissue was dehydrated in a graded series of alcohol and embedded in araldite CY212. The blocks were cut on microtome (LKB-8800 ultratome). Semithin sections (1  $\mu$ m thick) were routinely stained with toluidin blue for light microscopy. Ultrathin sections (60-80 nm thick) were contrasted with uranyl acetate and lead citrate and were examined with a transmission electron microscope (JEOL 1010) operating at 80 kV.

### *Measurement of Thickness of the Mucosal Biofilm*

10 sections from each group were measured by using an ocular micrometer for morphometric analysis. The thicknesses of averagely 10 sections from each group stained with PAS were measured by a ocular micrometer. The mean values of these measurement were calculated mean biofilm thickness were determined for each tonsil.

### *Statistical Analysis*

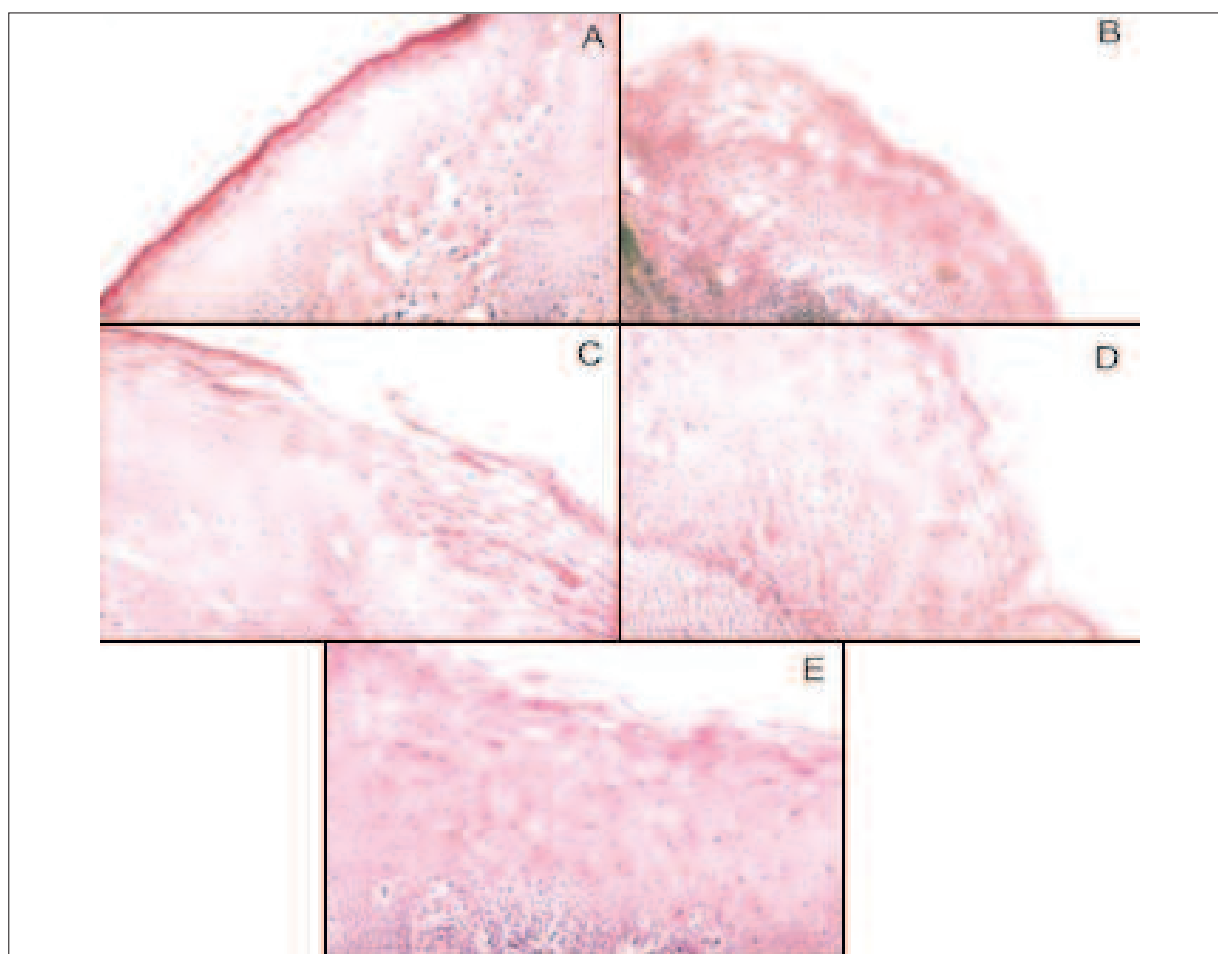
Statistical analysis was performed by using the Statistical Package for the Social Sciences for Windows (version 11.0; SPSS Inc., Chicago, IL, USA). The results were expressed as mean  $\pm$  standard deviation. The ANOVA test was used for the analysis of histopathological measurements. In the event of significant results, the Dunnette test was used for comparisons between the groups. *p* value < 0.05 was considered statistically significant.

## Results

### *Light Microscopy Analyses*

Control group; The thin layer of mucosal biofilm the tonsil epithelium with PAS staining, cells and bacteria are shown embedded in the tonsil mucosal biofilm layer (Figure 1A).

In the group tissue tonsil exposed 5 minute to NAC (Muconex<sup>®</sup>); the mucosal biofilm thin recorded as nearly disappear close to the normal surface epithelium. The areas on the tonsil biofilm of that section had the exopolysaccharide



**Figure 1.** Light microscopy images demonstrating bacterial biofilms on human tonsils. Control sample; bacterial pathogens were forming biofilm, mostly located on the outer surface of the tonsils. A multitude of bacteria attached to the outer surface of the tonsillar cells forming a multilayer is shown. Showing maximal 4+ observed **(A)** biofilm sample treated with NAC for 5 minute. Showing 3+ observed **(B)** NAC 10 minute. Showing 2+ observed **(C)** ASA 5 minute. Showing 2+ observed **(D)** ASA 10 minute. Showing 1+ observed **(E)** (All of figures PAS staining, original magnification,  $\times 80$ ).

matrix with embedded bacteria in an indentation at the opening of a crypt (Figure 1B).

In the group tissue tonsil exposed 10 minute to NAC (Muconex<sup>®</sup>); the mucosal biofilm layer view was recorded as dissappear also degeneration and necrotic cells were recorded on the surface epithelium (Figure 1C).

In the group tissue tonsil exposed 5 minute to ASA (Aspirin<sup>®</sup>); the reduction of the mucosal biofilm layer thickness on the surface epithelium was recorded (Figure 1D).

In the group tissue tonsil exposed 10 minute to ASA (Aspirin<sup>®</sup>); the mucosal biofilm layer was recorded as completely disappear with close to the normal surface epithelium (Figure 1E).

All of tonsil tissues were found to have biofilm on the tonsil surface, which was readily detected

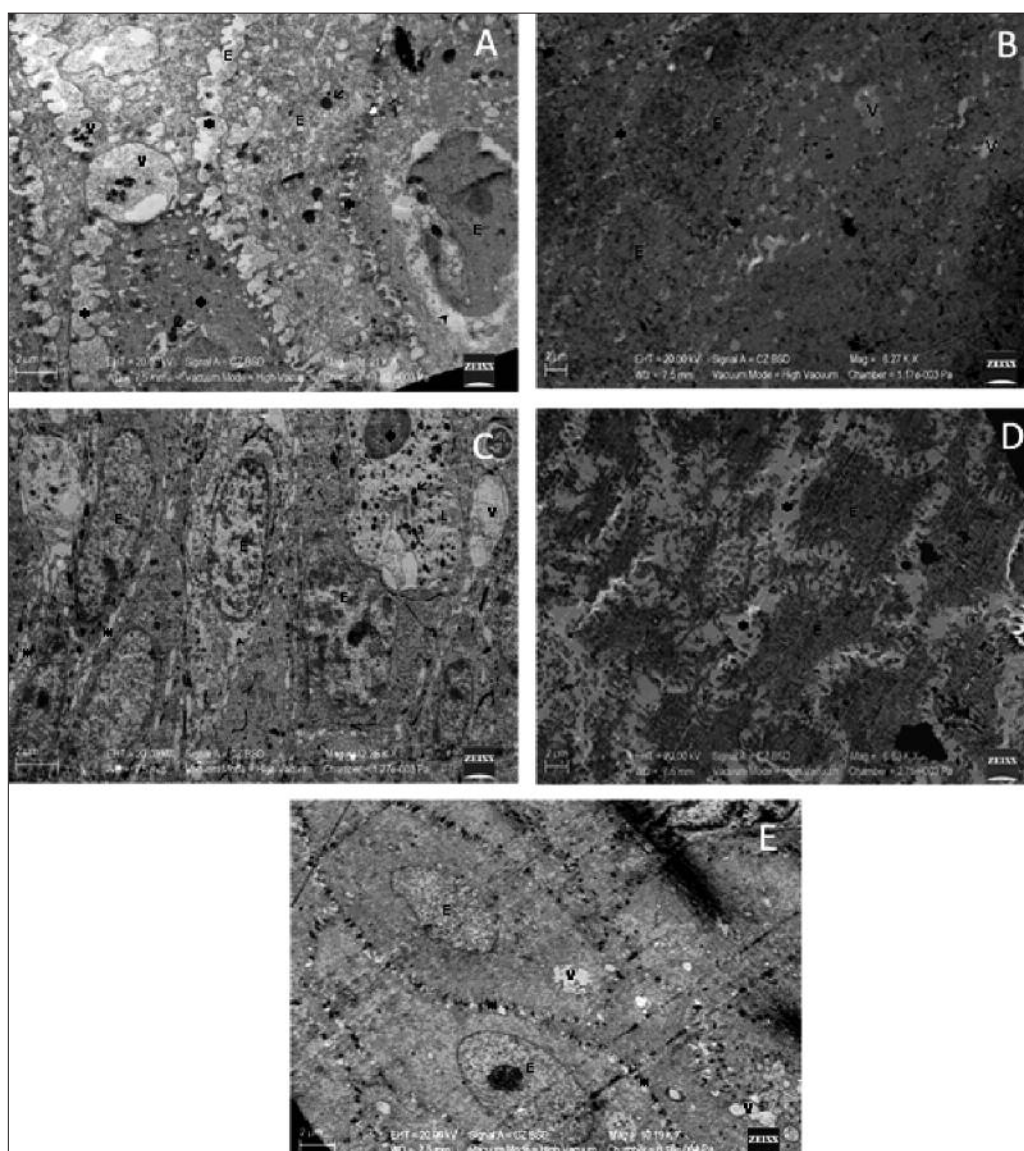
by PAS staining of the exopolysaccharide matrix in which the community of bacteria was located.

#### **Transmission Electron Microscopy Analyses**

Bacterial colonies were observed densely packed with a various morphological appearance; including rod-shaped, spherical profiles and a variety of capsular staining patterns. In close inspection, the bacteria were embedded in a homogeneous, amorphous background substance that was well preserved in solvent-processed tissues (Figure 2A-E).

#### **Statistical Analysis**

By light microscopy mean of biofilm layer thickness was  $8.39 \pm 1.74 \mu\text{m}$  before the therapeutic doses. The same figures were  $6.64 \pm 0.62$



**Figure 2.** Electron micrograph of a biofilm from human tonsillar crypt. A transmission electron microscopy (TEM) of a biofilm within tonsillar crypt. Control sample Showing maximal 4+ observed **(A)** biofilm sample treated with NAC for 5 minute. Showing 3+ observed **(B)** NAC 10 minute. Showing 2+ observed **(C)** ASA 5 minute. Showing 2+ observed **(D)** ASA 10 min. Showing 1+ observed **(E)** E: Epithelium cells, ◆: Epithelium cell with electron dense cytoplasm, >: Perinuclear ödem, \*: Extended lateral side connection fields, V: biofilm including vacuolar structure on connection unit, ↑: Intraepithelial biofilm (Uranil acetate-lead citrate. Original magnification; ×7000 A, ×6000 B, ×12000 C, ×6000 D, and ×10000 E, respectively).

$\mu\text{m}$  and  $4.72 \pm 0.63 \mu\text{m}$  in ASA – 5 minutes and ASA-10 minutes therapeutic doses groups, respectively. The thickness of biofilm layers were  $7.31 \pm 1.04 \mu\text{m}$  and  $6.13 \pm 1.03 \mu\text{m}$  in NAC – 5 minutes and NAC – 10 minutes, respectively. The mean of biofilm layer thickness for NAC-5 minutes did not show a significant difference compared with that of control group ( $p = 0.128$ ). However, the mean of biofilm layer thickness of NAC-10 ( $p = 0.008$ ), ASA -5 ( $p = 0.003$ ) and

ASA-10 ( $p \leq 0.0001$ ) groups showed significant differences with the control group, being the ASA-10 group most significant (Table I).

## Discussion

As a current theory, biofilm is one of the life models of bacteria<sup>9</sup>. Biofilm formations represent a serious clinical problem because it has been es-

**Table I.** Thickness of mucosal biofilm of tonsils.

Group (n= 10)	Mean $\pm$ SD ( $\mu$ m)	Minimum ( $\mu$ m)	Maximum ( $\mu$ m)
Control	8.39 $\pm$ 1.74	5.65	11.3
ASA-5	6.64 $\pm$ 0.62	6.03	7.61
ASA-10	4.72 $\pm$ 0.63	4.07	5.76
NAC-5	7.31 $\pm$ 1.04	5.65	9.11
NAC-10	6.13 $\pm$ 1.03	4.07	7.54

timated that more than 65% of all human bacterial infections involve biofilms<sup>10</sup>. However, biofilms particularly play a significant role for resistance against to medical treatment of chronic or recurrent tonsillitis<sup>11</sup>.

Biofilms have been recognized as a significant cause of persistent infections, including otitis media, cholesteatoma, dental plaque, pneumonitis resulting from cystic fibrosis, chronic cystitis, and also osteomyelitis during the last decade of human life<sup>9-14</sup>.

Chole and Faddis<sup>12</sup> used light microscopy and TEM to examine 19 removed tonsils, which have a history of repeated infection or airway obstruction from hypertrophy. The study shows that biofilm hasn't disposal with rinse or shaking in saline. The inhibitory effect on biofilm production of non-antibiotic compounds such as ASA<sup>14</sup> and NAC<sup>15</sup> have been reported. Recently, Ramage et al<sup>16</sup> have revealed the significance of applying for disinfectants on biofilm 30 min, 60 min, 90 min<sup>16</sup>. Our study confirms and extends the findings of the this study.

Studies, such as biofilm formation is useful at a general approach in tissues under oxidative stress to increase the accessibility of specific antioxidants, because the cells increases with a variety of mechanisms intracellular reduced glutathione (GSH) levels in response to various stress<sup>17</sup>. NAC has inhibitory effect on biofilm consisting on catheter of hemodialysis patients<sup>18</sup> and patients using voice prosthesis which has been displayed on biofilm<sup>19</sup>. Bacterial adhesion decreasing has been shown that depending on the dose of sodium salicylate<sup>20</sup>. The salicylic acid's effect is reducing production of slime factor on the biofilm created by *Staphylococcus epidermidis*, confirmed with electron microscopy<sup>21</sup>.

It is worth stressing that NAC not only reduced adhesion but in fact also detached adhered cells from a tonsil surface. NAC reduced the EPS production of cells that were incubated in the ab-

sence of energy. The more direct effects of NAC include a possible reaction of its sulfhydryl group with disulfide bonds in enzymes involved in EPS production or excretion, which renders these molecules less active, or competitive inhibition of cysteine utilization. The results of using NAC show a decrease *in vitro* biofilm formation in environments<sup>15</sup> and studies of salicylate were supported by shown the inhibiting production of biofilm<sup>22</sup>.

We found non-antibiotic compounds of ASA and NAC the reduces effect on tonsils mucosal biofilm formation in chronic or recurrent tonsillitis specimens. We found as effective the therapeutic doses of NAC and ASA against bacterial biofilm formation of analysed tonsil tissues at 10 patients. Our this study has demonstrated 2 major findings about the relationship of decrease to tonsil bacterial biofilm using NAC and ASA. First, ASA-10 were inhibitory found in the tonsil biofilm formation extensive search under a magnification of  $\times 80$ . Second, the effect of ASA were dose related at the biofilm formation, which confirms our hypothesis that decrease biofilm during the fixation process is required for detection of the bacterial biofilm by light microscopy. Staining of consecutive histologic sections with PAS and Giemsa allowed definitive localization of the bacteria in relation to the tonsil epithelium.

Recently, it has been reported that the newly introduced agents NAC and ASA active against biofilms as *in vitro*. Moreover, some aspirin concentrations (50 to 200 mg) producing significant levels of antibiofilm activity *in vitro* fall within the range of those frequently achieved by therapeutic doses of aspirin in humans<sup>16-23</sup>.

However, *in vitro* inhibitory effect of NAC and ASA hasn't been reported on the tonsil mucosal biofilm formation. Our study shows that ASA-10 reduces the biofilm formation.

## Conclusions

NAC and ASA can be considered for mitigating or preventive role on mucosal biofilm consisting chronic tonsillitis. Particularly, ASA therapeutic doses may be blocked or may occur later complications bring about at the early period of chronic tonsillitis of which reduction the number of antibiotic with the shortening at the treatment period. Thus, work power losses may able to minimized. Antibiofilm agents are needed more investigations for to evaluate in the ENT field.

### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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