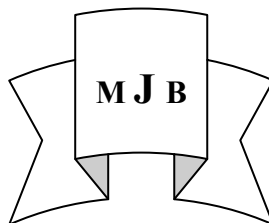


Apoptosis and Apoptotic Factors (BCL2 and Bax Protein) in Recurrent Benign Nasal Polyp

Musaab Habeeb Ali

Husam Majeed Hameed

College of Medicine ,Wassit University, Iraq.

E-mails: m_al_ameer@yahoo.comhusam_majeed@yahoo.com

Abstract

Apoptosis is an evolutionarily conserved form of cell death .In this respect, molecular characterization of the mechanisms involved in the regulation and execution of apoptosis could provide crucial information for understanding the pathogenesis of nasal polyps. The aims were as follow: 1. To study the percentage of apoptotic cells of nasal polyp by TUNEL assays (the recognized DNA fragmentation within the cells). 2. To study the role of versus reactions between anti apoptotic Bcl2 and pro apoptotic Bax proteins in surviving cells of nasal polyp. Polypectomy for 42 patient with recurrent nasal polypoidal masses was taken with a small piece of normal inferior turbinate as a control tissue & kept separately as biopsy for TUNEL assay for detection of percentage of apoptosis & for the detection of the percentage of Bcl2 & Bax protein immunohistochemical study. The Results showed that. the percentage of apoptotic cells in non nasal polyp tissue were significantly higher than the percentage of apoptotic cells in nasal polyp tissue (73.94 ± 15.12 , 18.86 ± 6.56), with $P\text{-value} < 0.001$). The percentage of Bcl2 protein in non nasal polyp tissue was significantly lower than the percentage of Bcl2 protein in nasal polyp tissue (20.25 ± 4.46 , 71.27 ± 17.26) respectively with $P\text{-value} < 0.001$) while the percentage of Bax protein in non nasal polyp tissue was significantly higher than the percentage of Bax protein in nasal polyp tissue (85.78 ± 5.42 , 33.14 ± 10.13) respectively, with $P\text{-value} < 0.001$. The apoptotic mechanism is lower in the recurrent nasal polyp tissue than in the normal (inferior turbinate) tissue. this may have a rule in the aetiopathology of the recurrence of nasal polyps.

الاستماتة و عوامل الاستماتة (بروتين BCL2 و Bax) في الزوائد اللحمية الحميدة الراجعة

الخلاصة

الاستماتة هي عملية تموت مبرمج لموت الخلايا و دراسة هذ الميكانيكية قد تزودنا بمعلومات اساسية لفهم امراضية الزوائد اللحمية في الانف. تهدف هذه الدراسة الى: اولا دراسة النسبة المئوية لاستماتة خلايا الزوائد اللحمية للمرضى بأستخدام طريقة التونيل و التي تعمل على تحديد اماكن تقطع شريط الدنا الناتجة عن عملية الاستماتة فقط. ثانيا دراسة بعض البروتينات المضادة للاستماتة و البروتينات المحفزة للاستماتة لتحديد اسباب الخلل ان وجد.

تناولت الدراسة ٤٢ مريضاً يعانون من زوائد لحمية راجعة تم استئصالها و من ضمنها جزء من نسيج طبيعي لغرض السيطرة و المقارنة وحفظها لحين استخدامها لقياس النسبة المئوية للاستماتة ومن ثم دراسة العوامل المؤثرة عليها. وكانت النتائج كالآتي : النسبة المئوية لاستماتة النسيج الطبيعي اعلى من النسبة المئوية لاستماتة خلايا الزوائد اللحمية للمرضى (73.94 ± 15.12 , 18.86 ± 6.56) و بمعيارية ($P\text{-value} < 0.001$). و النسبة المئوية للبروتين المضاد للاستماتة في النسيج الطبيعي اقل من النسبة المئوية لبروتين خلايا الزوائد اللحمية (20.25 ± 4.46 , 71.27 ± 17.26) و بمعيارية ($P\text{-value} < 0.001$). و النسبة المئوية للبروتين المحفز لاستماتة للخلايا الطبيعية اعلى من النسبة المئوية للبروتين في النسيج غير الطبيعي (85.78 ± 5.42 , 33.14 ± 10.13) و بمعيارية ($P\text{-value} < 0.001$). ومن هذا يلاحظ ان عملية الاستماتة في النسيج الغير طبيعي (الزوائد اللحمية) اقل من عملية الاستماتة في النسيج الطبيعي و هذا قد يكون سبب من اسبابا نشوء المرض و اعادة الإصابة به.

Introduction

Nasal polyposis typically consists of an underlying edematous or myxomatous stroma with an overlying intact respiratory epithelium, which shows various degrees of tissue remodeling in the epithelium, glands, connective tissues, and vessels [1, 2]. Although the pathogenesis of this nasal disease has recently been dealt with in numerous articles, the etiology and pathogenesis of nasal polyps are still controversial [3]. Recently it has been suggested that a fungus-mediated process is the primary cause of chronic respiratory sinusitis with and without polyps [4]. Much evidence has shown that the apoptotic mechanism induces the secondary changes in chronic inflammation of various organs, including epithelial hyperplasia or tissue remodeling. In this respect, molecular characterization of the mechanisms involved in the regulation and execution of apoptosis could provide crucial information for understanding the pathogenesis of nasal polyps [5]. Apoptosis is an evolutionarily conserved form of cell death that was first described by Kerr et al in 1972 [6]. It is essential for successful embryonic development and maintains normal cellular homeostasis in adult organisms [7].

Apoptosis pathways

Apoptosis is an evolutionarily conserved form of cell death that was first described by Kerr et al in 1972 [8]. It is essential for successful embryonic development and maintains normal cellular homeostasis in adult organisms. Any abnormality in apoptotic pathway suggests that perturbation of cellular homeostasis can be a primary pathological event that results in disease [9]. There is now compelling evidence that insufficient apoptosis can result in cancer or autoimmunity [10], whereas

accelerated cell death is evident in degenerative diseases, immunodeficiency and infertility [11-13].

The extrinsic pathway can be initiated by one of several cell surface death receptors when bound by the appropriate ligand [10]. TNF receptor 1 (TNFR-1) and Fas receptors contain death domains (DDs) and recruit the DD-containing adaptor molecules, TNFR-1-associated death domain (TRADD), and Fas-associated death domain (FADD), respectively. Homotypic interaction between the DDs of Fas and FADD induces the recruitment and self-activation of pro-caspase-8 [8, 14, 15]. In TNF signaling, TRADD recruits FADD after formation and release of a TNFR-1 complex to initiate pro-caspase-8 activation [12, 16]. The receptors for TNF-related apoptosis-inducing ligand (TRAIL), TRAIL-R1 (also known as death receptor-5, DR-5), also recruit and activate pro-caspase-8 [17] in a FADD-dependent manner [18].

The intrinsic pathway is characterized by the permeabilization of the outer mitochondrial membrane and the release of several pro-apoptotic factors into the cytosol. These factors include cytochrome c [19, 20], Smac/Diablo [21, 22], apoptosis-inducing factor (AIF) [23], endonuclease G (24, 25), and HtrA2/Omi [26]. The release of these mediators is regulated by the Bcl-2 family [27]. Once released, cytochrome c binds to an adaptor protein, Apaf-1, which self-oligomerizes and recruits pro-caspase-9 to form the apoptosome complex [28]. This promotes the autoprocessing of caspase-9, which in turn recruits and cleaves pro-caspase-3, which degrades proteolytically targeted substrates and activates DNases [29].

In this study we assessed the Bcl2 and Bax protein for the following reasons:-

The functions of Bcl2 protein are:

1. The sub cellular localization of Bcl2 protein may provide a clue to its function that Bcl2 has been found to be associated with mitochondrial, nuclear and endoplasmic membrane [30] So, the Bcl2 protein may be important for membrane lipid integrity by suppressing the generation of reactive oxygen species [31].
 2. Bcl2 protein can inhibit the direct effect of pre apoptotic (stimulator) Bax proteins by (Bcl2-bax protein interaction) to form heterodimer complex [32].
 3. Over expressions of Bcl2 blocks TNF-related apoptosis inducing ligand (TRAIL). TRAIL triggers apoptosis by binding to its two pre apoptotic receptor DR4 and DR5 and via cleavage of caspase 8, 9, 7, 3 and BID, release cytochrome c from the mitochondria and cleavage of poly (ADP-ribose) polymerase (PARP) [33].
 4. When Bcl2 protein increase it can block p53 induced apoptosis [34].
- While the mechanism of action Bax protein on mitochondrial membrane is still ambiguous, Bax which form trans membrane channel protein causes change in membrane potential that leads to release of cytochrome c and other pro apoptotic that causes caspase activation and hence activation of programmed cell death [35]. The action of pro apoptotic Bax protein on mitochondrial membrane is to open the mitochondrial permeability transition pore of outer membrane lead to release of mitochondrial apoptotic proteins cytochrome c, Apaf-1(apoptotic activator factor-1)and Smac/Diablo protein (Second mitochondrial derived activator of caspase/Direct IAP

Binding protein with low isoelectric point) [36].

Objectives

- 1-To study the percentage of apoptotic cells of nasal polyp by TUNEL assay (the recognized the DNA fragmentation within the cells).
- 2-To study the role of versus reactions between anti apoptotic Bcl2 and pro apoptotic Bax proteins in nasal polyp mass.

Patients and Methods

Polypectomy was done for a 42 patient with recurrent nasal polyp during the period from April (2006) to April (2008). The polypoidal masses were taken with a small piece of normal inferior turbinate as a control and kept separately as biopsy for TUNEL assay for detection of percentage of apoptosis and immunohistochemical study for the detection of the percentage of Bcl2 and Bax protein.

The nasal polyp mass for each individual was prepared to study the percentage of apoptosis, anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein through immunohistochemistry.

Immunohistochemistry procedure:

TUNEL Staining:

Procedure: (According to the guide of *Promega* com. Kit. USA)

1. The deparaffinize and rehydrate tissue sections were done by washing specimen in two changes of xylene for 5 min each, two changes of absolute ethanol for 5 min each, one wash in 95% and 70% ethanol for 3 min each and finally, one wash in phosphate puffer saline (PBS) for 5 min.
2. Applying proteinase K were done (100 µl of 2.5 to 5 µg/ml) directly to the tissue sample, incubate for 15 min at room temperature, then washing four times in distilled water for 2 min each achieved.

3. Endogenous peroxide was quenched by incubation in 3% hydrogen peroxide in PBS for 5 min at room temperature. Then washing twice done in PBS for 5 min each.

4. Removal of all excess liquid from slide was done by appl 50 µl of equilibration buffer to each sample, cover with a plastic cover slip and incubate at room temperature for up to 30 min.

5. The removal of equilibration buffer done by tapping off liquid, apply 25 µl of TdT solution (20 U/100 µl) made up in reaction buffer. Cover with plastic cover slip and incubate in a humid chamber at 37°C for 1 h.

6. After that labeling reaction terminated by incubating the section in stop buffer for 15 min at room temperature. Wash thrice in PBS for 5 min each wash.

7. Peroxidase conjugated antidigoxigenin antibody For indirect TUNEL staining, and incubated sections for 30 min at room temperature. Wash samples thrice with PBS for 5 min each.

8. Then the addition of 150 µl of DAB solution to sections was done and let the color development proceed for 5 min at room temperature. Wash samples four times with distilled water for 5 min each.

9. Counter stain sections for 3 min in methyl green solution. Wash sections with three changes of water followed by three changes of 100% butanol.

10. The washing in three changes of xylene and mount sections under glass cover slip done, using an appropriate mounting medium. View samples under light microscope (show figure 1 A and B).

Bcl2 and Bax proteins staining

Procedure: (According to the guide of Chemicon com kit USA)

1. Dewaxing: paraffin embedded sections were placed inside a hot air oven at 65°C overnight, then dipped in

xylene and ethanol containing jars in the following order:

a. Xylene : 5 minutes.

b. Fresh xylene : 5 minutes.

c. Absolute ethanol: 5 minutes.

d. Fresh absolute ethanol: 5 minutes.

e. Ethanol (95%): 5 minutes.

f. Ethanol (70%): 5 minutes.

2. Slides were washed in distilled water for 5 minutes then drained and blotted gently.

3. 100 µl of a protein-blocking reagent was placed onto the section and incubated for 10 minutes in a humid chamber at room temperature. Then slides were drained and blotted gently.

4. 100 µl of diluted primary antibody was placed onto the section and incubated for 1 hour at 37°C in a humid chamber. After incubation, the slides were drained and blotted gently.

5. Slides were rinsed with PBS-Tween for 5 minutes, then drained and blotted gently.

6. 100 µl of diluted secondary antibody was placed onto the section and incubated for 30 minutes at 37°C in humid chamber. Slides were drained and blotted gently.

7. Slides were rinsed with PBS-Tween for 5 minutes, then drained and blotted gently.

8. 100 µl of diluted streptavidin-alkaline phosphatase conjugate was placed onto the section and incubated for 20 minutes at 37°C in humid chamber. Slides were drained and blotted gently.

9. Slides were rinsed with PBS-Tween for 5 minutes, slides were drained and blot gently, then 100 µl of diluted activation buffer was placed on the section and incubated for 5 minutes then drained and blot gently.

10. One hundred microliter (100 µl) of the BCIP/NBT substrate was placed onto the section and incubated for 10 minutes at room temperature. .

11. Slides were washed in running water for 5 minutes and then drained and blotted gently.

12. 100 µl of counter stain (nuclear fast red) was placed onto the section and incubated for 1 minute at room temperature. Slides were drained and blotted gently.

13. Slides were washed in distilled water then dehydrated by placing them in ethanol and xylene in the following order:

- a. 70% ethanol for 3 minutes.
- b. 95% ethanol for 3 minutes.
- c. Absolute ethanol for 5 minutes.
- d. Xylene for 5 minutes.
- e. Fresh xylene for 5 minutes.

14. A drop of mounting medium (DPX) was placed onto the xylene-wet section by using a xylene-moist cotton swab and the section was quickly covered with a cover slip. Slides were let to dry (show figure 2 C and D, figure 3 E and F).

Results

The percentage of apoptotic cells in non nasal polyp tissue were significantly higher than the percentage of apoptotic cells in nasal polyp tissue (73.94 ± 15.12 , 18.86 ± 6.56) respectively, with (P-value < 0.001) show table (1)

Table 1 Comparison between percentage of apoptotic cells of non nasal polyp tissue (normal tissue) and nasal polyp tissue

Group	% of apoptotic cells
Controls (non nasal polyp tissue)	73.94 ± 15.12
Patients (nasal polyp tissue)	18.86 ± 6.56
P-value	< 0.001

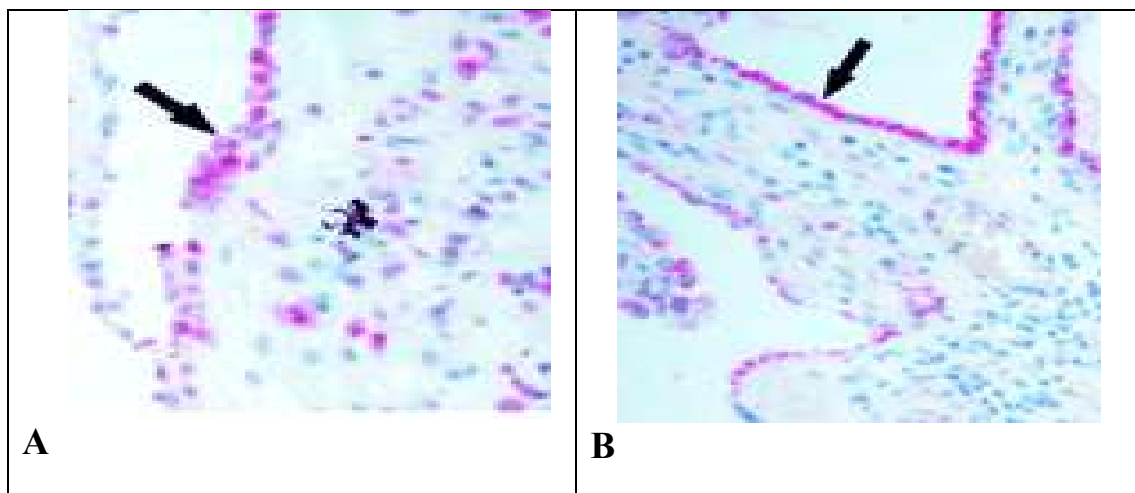


Figure 1 Immunohistochemistry staining of TUNEL of tissue by peroxidase (red), counter stained with Meyer's hematoxylin (blue). A: Nasal turbinate, black arrow show cells stained with DAB stains (showing highly apoptosis percent) within normal tissue and cells stained with hematoxylin stains. B: Nasal polyp tissue black arrow show cells stained with DAB stains (showing low apoptosis percent) and cells stained with hematoxylin. A and B high power magnification of 1000X.

The percentage of Bcl2 protein in non nasal polyp tissue were significantly lower than the percentage

of Bcl2 protein in nasal polyp tissue (20.25 ± 4.46 , 71.27 ± 17.26) respectively, with (P-value < 0.001),

while the percentage of Bax protein in non nasal polyp tissue were significantly higher than the percentage of Bax protein in nasal polyp tissue) (85.78 ± 5.42 , 33.14 ± 10.13)

respectively, with (P-value <0.001) show table (2).

The results gain from this study were analyzed using independent sample T test.

Table 2 Comparison between % of Bcl2 and Bax in non nasal polyp and nasal polyp tissue

Group	% Bcl2	% Bax
Controls (non nasal polyp tissue)	20.25 ± 4.46	85.78 ± 5.42
Patients (nasal polyp tissue)	71.27 ± 17.26	33.14 ± 10.13
P-value	<0.001	<0.001

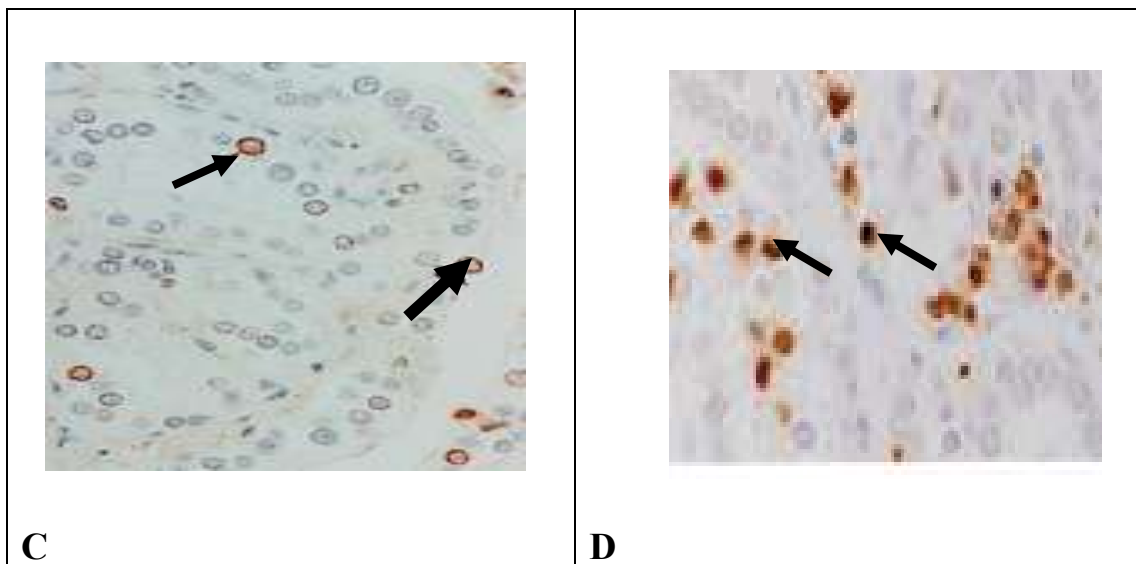


Figure 2 Immunohistochemistry staining of Bcl2 of tissue by peroxidase/DAB (brown) (showing low distribution), counter stained with meyer's hematoxylin (blue). C: Nasal turbinate, black arrow show cells stained with DAB stains, with cells stained with hematoxylin stains. D: Nasal polyp tissue black arrow show cells stained with DAB stains (showing high distribution) with cells stained with hematoxylin. C and D high power magnification of 1000X.

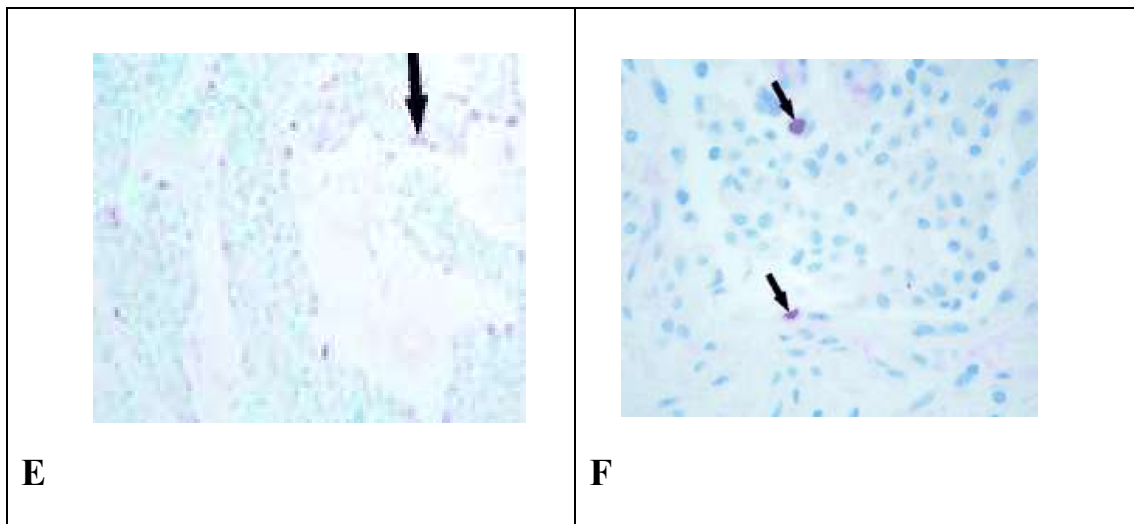


Figure 3 Immunohistochemistry staining of BAX of tissue by Alkaline phosphatase/chromogen fast A, B (red) (showing high distribution), counter stained with meyer's hematoxylin (blue). E: Nasal turbinate, black arrow show cells stained with chromogen fast A, B stains, with cells stained with hematoxylin stains. F: Nasal polyp tissue black arrow show cells stained with chromogen fast A, B stains (showing low distribution) with cells stained with hematoxylin. E and F high power magnification of 1000X.

Discussion

In this study, there was a decrease in the recurrent nasal polyp apoptosis (73.94 ± 15.12 , 18.86 ± 6.56) respectively, with (P-value < 0.001), and this agree with the studies of M. L.Kowalski, J.Grzegorzcyk and R.Pawliczak [37] where they found a significant decrease in percentage of nasal polyp apoptosis in compared with tissue of inferior turbinate, which indicate the important role of apoptotic mechanism in nasal polyp recurrence.

The result of these studies indicates that in nasal polyp the cells have a specific pathway for long term survival and this can be explained by the role of anti apoptotic Bcl2 protein and pro apoptotic Bax protein in nasal polyp mass.

Inhibition of nasal polyp apoptosis is playing an important role in the recurrence of nasal polyps. In this study there was significantly decrease in percentage of Bcl2 protein in non nasal polyp tissue in compare with nasal polyp tissue ($20.25 \pm 4.46.12$, 71.27 ± 17.26) respectively, with (P-

value < 0.001), while the percentage of Bax protein in non nasal polyp tissue were significantly higher than the percentage of Bax protein in nasal polyp tissue (85.78 ± 5.42 , 33.14 ± 10.13) respectively, with (P-value < 0.001). The imbalances between Bcl2/Bax proteins explain one of molecular mechanism for inhibition of apoptosis in nasal polyp there was no studies working on the same parameters of apoptosis proteins and because for selecting these parameters were explained in the introduction

The role of apoptosis had been studied by many researchers through the different path ways of apoptosis, most of them reach the same results that is apoptosis was low in nasal polyps and all of these studies were done on a nasal polyp without mentioning whether it is just a polyp or recurrent nasal polyp while in our study all the polyp tissues were taken from patients that sustained a previous simple nasal polypectomy (under general or local anesthesia)

Cho SH. *et al* (2008) [38] show that cIAP1, cIAP2, XIAP, and caspase 3 may regulate the homeostasis of normal nasal mucosa, whereas cIAP2, XIAP, and caspase 3 may take part in the pathogenesis of nasal polyps.

Qiu ZF *et al* (2008) [39] also showed increased expression of survivin in nasal polyps at both the mRNA and the protein levels, the elevated expression of survivin might play an important role of development in nasal polyps.

Garavello W, *et al* (2005) [40] they work on P53 and they conclude P53 play important role in nasal polyp persistence.

Li Q, *et al* (2005) [41] showed the downwards expression of Fas and the upwards expression of FasL in nasal polyps may inhibit the cellular apoptosis and the enhanced hyperplasia.

So, from the above we found that by all the pathways of apoptosis extrinsic and intrinsic pathway apoptosis decreased in the nasal polyps.

Conclusion

The apoptotic mechanism is lower in the recurrent nasal polyp tissue than in the normal (inferior turbinate) tissue, this may have a role in the aetiopathology of the recurrence of nasal polyps.

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