



Evaluation of the effect of intranasal infiltrated botulinum toxin-A on a model of allergic rhinitis in rabbits: An Experimental Study[☆]



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ABSTRACT

Introduction: Allergic Rhinitis (AR) affects 20–40% of the global population and its prevalence increases. Medical treatment and immunotherapy could be used in AR management. But they are not definitive solution. Medical treatment must be used a long time and has side effects. Immunotherapy cannot be applied to every patient and it also takes a long time. The aim of this study is to evaluate symptomatic and histopathological effects of intranasal infiltrated Botulinum Toxin-A (Btx-A) on an animal model of AR.

Material-Method: 15 rabbits were divided into 3 groups as control, disease and treatment. AR was formed in disease and treatment groups by intraperitoneal and intranasal ovalbumine. Allergic symptoms were observed and serum IgE levels were estimated to prove forming of AR. Btx-A was infiltrated in inferior turbinates of rabbits in treatment group. Rabbits were sacrificed on 32nd day. Paranasal structures were dissected and investigated histopathologically.

Results: Serous nasal discharge and sneezing were observed after ovalbumine applying in disease and treatment groups. Serum IgE levels on 21st day were higher than 1st day and this difference was significant statistically in disease and treatment groups. Serous discharge and sneezing decreased after Btx-A infiltration in treatment group. In histopathological examination, there were significant difference between disease and treatment group in terms of some histopathological findings.

Conclusion: Considering the effect of Btx-A on AR in animal, it can be said that Btx-A can decrease symptoms and reorganize histopathological findings of AR.

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1. Introduction

Allergic rhinitis (AR) is an inflammatory disease characterized by episodes of sneezing, pruritus, rhinorrhea, nasal congestion and lacrimation that occur after exposure to the causative allergen. Itching can be seen in palate, throat, eyes, and ears. AR is caused by contact with allergen like pollen, mold and mite and an inflammation occurs in nasal mucosa. AR is a common disease. It affects 20–40% of the global population and its prevalence

increases [1]. AR is responsible for the etiology of sinusitis, tonsillopharyngitis, otitis media, conjunctivitis and decreases response to treatment and quality of life [2].

The first and most effective treatment step is to avoid the provoking allergen. In addition to, intranasal and systemic steroid, antihistamines, decongestant, cromolyn sodium, anti-leukotrienes, anticholinergics, anti-IgE agents, capsaicin and intranasal saline could be used in the treatment, when avoiding the allergen is not enough or effective [3]. Although medical treatments reduce symptoms, it is not curative. Furthermore; patients do not want to use medications a long time and medical agents have some disadvantages like side effect and high cost. Another option for the treatment is immunotherapy that can change the natural course of the disease. It also has disadvantages. For example, it cannot be apply every patient and is not successful in every intervention. It is troublesome for both patient and doctor due to need a long-period

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(4 years) and has the risk of anaphylaxis [4]. Surgical treatments like septoplasty, turbinateplasty, excision of nasal polyps are helpful to decrease symptoms but they do not cure AR and medical treatment is needed to continue. When considering all of them; treatment options that are easy-to-use, provide to increase quality of life, have less side effect and cost are needed.

Botulinum toxin (Btx-A) is a protein produced by an anaerobic bacterium, *Clostridium botulinum*. Btx-A is often used in otolaryngology and a research area for the treatment of AR. Btx-A causes inhibition of acetylcholine (ACh) release from cholinergic nerves in nasal mucosa and preganglionic nerves in sphenopalatine ganglion. In addition, it can cause apoptosis induction in nasal glands. Through this mechanism, Btx-A can reduce parasympathetic effects in nasal cavity [5]. There are some reports about the effects of topical or injectable form of Btx-A to nasal mucosa in humans with vasomotor rhinitis or AR. Besides, the effect of topical Btx-A is investigated in AR created animal models [5–8]. Therefore, there is no study investigating the histopathological effect of Btx-A that infiltrated in inferior turbinate. The effects of infiltrated Btx-A in AR created rabbits were investigated in this study.

2. Material and method

After the animal experimentation ethics committee approval, 15 male rabbits from New Zealand race (Experimental and Clinical Research Center, Erciyes University, Turkey) were enrolled in the study. They were 20–24 weeks old and their weight ranged 3000–3500 gr. They were fed a standard laboratory diet and kept in a 21 ± 2 °C degree-environment and 50% relative humidity with 12-hour light and dark cycle (07.00–19.00). They were allowed to eat and drink as much as they want in the experiment period by mixed feed in the form of pellets. Experimental procedures were applied in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) principles of international animal research.

Rabbits were divided into 3 groups as:

- Control group (CG): 5 rabbits applied only serum physiologic (SP)
- Disease group (DG): 5 rabbits created AR without treatment
- Treatment group (TG): 5 rabbits created AR and applied infiltrated Btx-A

2.1. Forming allergic rhinitis

1st stage: A solution was prepared with 100 ml 0.9% SP, 30 mg ovalbumin (OVA, grade V, Sigma, St. Louis, MO, USA) and 3 gr aluminum hydroxide. 5 ml from this solution was injected every two days for 14 days intraperitoneally (i.p.) to rabbits in DG and TG.

2nd stage: Another solution was prepared with 10 ml SP and 1 gr OVA. 0.1 ml solution was dropped in the two nostrils of rabbits in DG and TG once every day in the same time from 14th day for 18 days.

5 ml SP was injected 7 times i.p. to rabbits in CG once in the same time and in every two days. 0.1 ml SP was dropped in the two nostrils of rabbits in CG once every day in the same time from 14th day for 18 days.

Serum specific OVA IgE values were measured in the 1st and 21st days of the study. Nasal examination was done on every rabbit before the beginning of experiment and intranasal application. When observed increasing in serum specific OVA IgE, serous nasal discharge and nose scratching in DG and TG group, this was accepted as successful in terms of forming AR model.

2.2. Btx-A application

In the 21st day of the study, 10 U (5 U for each side) Btx-A was infiltrated in inferior turbinates of rabbits in TG bilaterally (Image 1).

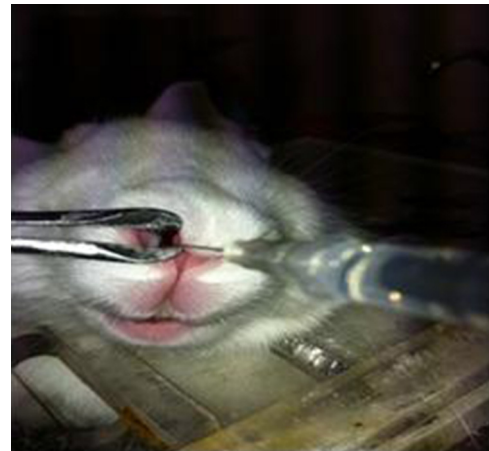


Image 1. Infiltration of botulinum toxin-A in inferior turbinate of a rabbit.

It was done under anesthesia with 35 mg/kg ketamin and 5 mg/kg xylazine. Btx-A was diluted by SP as 50 U/ml and applied 0.2 ml by a 27 G injector. After application, there was no complication and side effect except a minimal bleeding that was observed in every rabbit. All bleedings stopped spontaneously without tampon.

2.3. Histological evaluation

All rabbits were sacrificed in the 32nd day of the study. Nasal cavity, nasal septum, paranasal sinuses and turbinate of rabbits were removed in a piece. Sections were done from behind the incisors to the end of the hard palate.

Tissues were fixed in 10% formalin solution for 15 days. Following that, they were decalcified by 5% nitric acid solution. Tissues were washed by tap water for 24 h. Mucosal tissues were dehydrated in graded alcohol series and embedded in paraffin. A transverse section was cut (4 m) and stained with hematoxylin Hematoxylin–Eosin (HE), Alcian blue (AB), periodic acid shift (PAS) and Masson's trichrome (MT). They were evaluated by Olympus BX-51 photomicroscope and photos were taken.

The histological findings were scored in terms of these features: squamous cell metaplasia, Goblet cell proliferation, connective tissue increasing, vascular proliferation and congestion, inflammatory cell and eosinophils infiltration and chondrocyte hypertrophy. Scoring was done semiquantitatively according to previous studies [9,10]. When there was no change, it was scored as 0 point; minimal change was scored as 1 point; prominent change was scored as 2 point, severe change was scored as 3 point. For statistical analyzing, rabbits in every group were divided into two groups according to score. If score was under 1.5, it was included in none/mild change group. When score was more than 1.5, it was included in prominent/severe change group.

2.4. Statistical analysis

Data were analyzed by SPSS 22.0 package program. Paired Sample Test was used for repetitive data. Fisher's Exact Chi-square Test with Monte Carlo Correction was used to compare categorical data. When *p* value was under 0.05, this was accepted as significant statistically. If there was a significant difference between 3 groups, pairways comparisons were done to define different group.

3. Findings

Nose examinations were normal in all rabbits before the experiment. Serous nasal discharge was observed in DG and TG from the first i.p. OVA application. This discharge increased after

Table 1

Comparing serum IgE levels on 1st and 21st days.

	Serum IgE level (k U/l) 1st day (Mean ± SD)	Serum IgE level (k U/l) 21st day (Mean ± SD)	P
Control Group	0.0980 ± 0.09338	0.0420 ± 0.09391	0.368
Allergic Rhinitis Group	0.0820 ± 0.07563	2.1460 ± 0.67263	0.002
Treatment Group	0.0620 ± 0.08672	1.6440 ± 0.74557	0.006

p value was estimated by Paired Sample Test.

intranasal OVA application. A decreasing was observed in nasal discharge in TG after Btx-A infiltration. It completely disappeared in 3 days. There was no discharge in the following days. There was no change in CG.

Serum IgE levels of DG and TG increased on 21st day. These changes were significant statistically (Table 1).

AR-related histopathological changes (Goblet cell increasing, connective tissue increasing, vascular proliferation and congestion, inflammatory cell and eosinophils infiltration, chondocyte

hypertrophy) were observed in DG. But these changes were similar with CG and TG after Btx-A infiltration (Table 2).

There was no significant difference in terms of squamous metaplasia, Goblet cell proliferation, inflammatory cell and eosinophils infiltration between 3 groups ($p > 0.05$). But in DG, scores about these features were higher. These scores were similar in CG and TG (Table 3) (Images 2–4).

There were significant differences between 3 groups in terms of connective tissue proliferation, vascular proliferation, congestion and chondrocyte hypertrophy ($p < 0.05$). In pairways comparisons, DG was different from the others. Results of CG and TG were similar (Table 3) (Image 5).

4. Discussion

Immunohistochemical studies about changes in AR show increasing number of eosinophils, basophils and mast cells in lamina propria and surface epithelium of nasal mucosa. In addition to; structural changes like thickening of the basement membrane, decreasing number of epithelial cells, epithelial metaplasia, mucous gland hyperplasia, Goblet cell hyperplasia,

Table 2

Histopathological semiquantitative scores of rabbits.

	CG1	CG2	CG3	CG4	CG5	DG1	DG2	DG3	DG4	DG5	TG1	TG2	TG3	TG4	TG5
SCM	0	0	0	0	0	2	0	2	1	2	0	0	1	0	0
GCP	0	0	0	0	0	2	2	1	1	1	0	0	0	0	1
CTP	0	0	0	0	0	2	2	2	3	3	1	0	1	0	0
VCP	1	1	1	0	0	3	3	3	3	2	1	1	0	1	0
ICI	0	0	0	0	0	2	2	1	2	1	0	0	0	0	0
CH	1	1	0	0	0	3	2	3	2	2	1	2	0	1	0

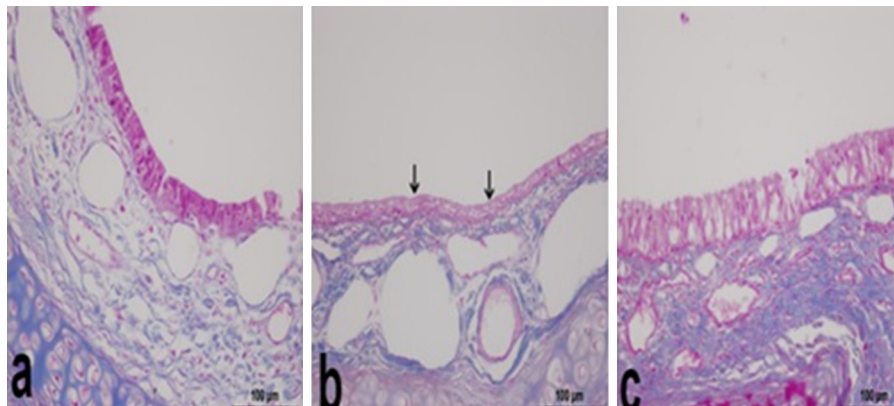
CG: Control Group; DG: Disease Group; TG: Treatment Group; SCM: Squamous cell metaplasia; GCP: Goblet cell proliferation; CTP: Connective tissue increment; VCP: Vascular congestion and proliferation; ICI: Inflammatory cell infiltration; CH: Chondrocyte hypertrophy; 0: No change; 1: Minimal Change; 2: Prominent change; 3: Severe change.

Table 3

Rabbit numbers according to score and comparing these results.

	Control Group		Allergic Rhinitis Group		Btx-A Treatment Group		<i>P</i>
	None-Mild (Score < 1.5)	Prominent-Severe (Score > 1.5)	None-Mild (Score < 1.5)	Prominent-Severe (Score > 1.5)	None-Mild (Score < 1.5)	Prominent-Severe (Score > 1.5)	
SM	5	0	2	3	5	0	0.067
GCP	5	0	3	2	5	0	0.278
ICI	5	0	2	3	5	0	0.067
CTI	5	0	0	5	5	0	0.007
VCP	5	0	0	5	5	0	0.067
CH	5	0	0	5	4	1	0.007

SM: Squamous Metaplasia; GCP: Goblet Cell Proliferation; ICI: Inflammatory and Eosinophils Infiltration; CTI: Connective Tissue Increasing; VCP: Vascular Congestion and Proliferation; CH: Chondrocyte Hypertrophy.

**Image 2.** Squamous cell hyperplasia with Masson's trichrome staining.

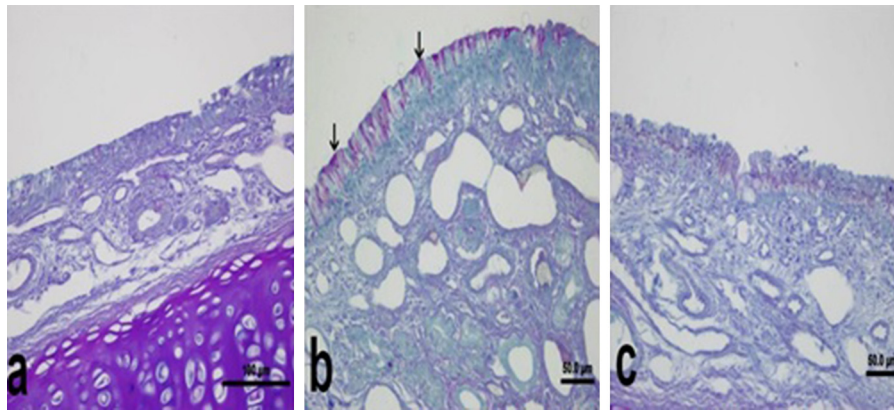


Image 3. Goblet cell proliferation with Alcian Blue and PAS staining. Arrows indicate Goblet cells in surface epithelium in allergic rhinitis group.

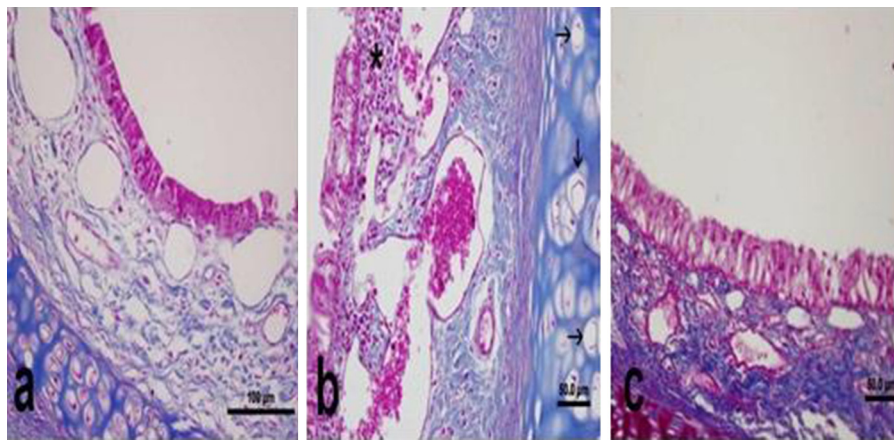


Image 4. Inflammatory cell infiltration (*), chondrocyte hypertrophy (arrows), congestion and connective tissue increasing are seen in allergic rhinitis group. Masson's trichrome staining.

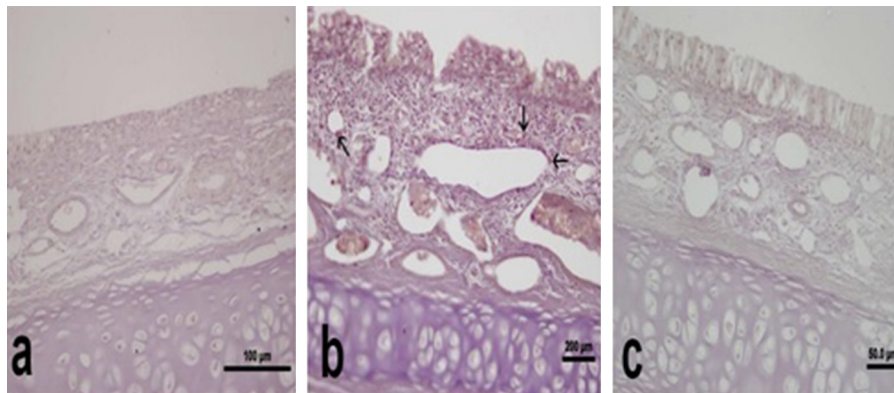


Image 5. A lot of eosinophils (arrows) are seen in allergic rhinitis group.

vascular proliferation and chondrocyte hypertrophy are also reported in AR [11]. There are some studies accomplished AR created rats showed eosinophils proliferation, vascular dilatation, congestion, edema and serous gland hyperplasia in nasal mucosa of rats [7,12]. Connective tissue proliferation, vascular proliferation and congestion, chondrocyte hypertrophy were showed in DG in this study and these changes were significant statistically. This also showed that the study is successful in terms of creating AR model in rabbits.

Prevention of sensitivity development in atopic people (primary prevention) is impossible today. It is also very difficult to avoid the onset of AR in sensitivity-developed people (secondary

prevention) [1]. Tertiary prevention (avoid allergens in patients have AR to prevent development of symptoms) is not enough most of the time [13]. While a treatment is arranged an AR patient, parameters like drug activity and safety, cost-benefit ratio, patient preference, aim of the treatment, severity of disease, patient compliance with the recommendations and comorbid diseases must be considered and optimal treatment should be given. When a drug is not effective or could not be used for a reason, alternative methods can be used. One of them is immunotherapy that can be curative. Although treatment response is very good in AR patients with single-pollen allergy, successful rate in multi-pollen allergy is lower [14–16].

There are different application methods about immunotherapy. Subcutaneous application is a hard and risky method. It takes a long time and the risk of anaphylaxis between sessions restricts its use [4]. Sublingual way has emerged because of risks of subcutaneous application. But there are some reports about failure of this method [4,17].

Complementary and alternative methods like intranasal phototherapy, herbal medicine and acupuncture is commonly used in AR. Therefore, their efficiency is not based on evidence and not supported last evidence [18]. There is no information about routine clinical use of these methods in recent guidelines [13]. Researches continue about this area.

Btx is the first microbial protein used in humans for the treatment. There are a lot of clinical application area about Btx and new studies continue in different pathological conditions [19]. The actual mechanism of action of Btx is blocking release of Ach from sympathetic cholinergic and parasympathetic neurons [20,21]. It is used in the treatment of strabismus, hemifacial spasm, spasmodic dysphonia, spasticity, distoni, salivary fistulas, removal of wrinkles and Frey Syndrome [21–23]. Because it is also effective in cholinergic autonomous nerves, it is thought that it can be used in situations with glandular hypersecretion like rhinorrhea and sialorrhoea [24].

Use of Btx-A in symptomatic treatment of AR has been investigated [5–7,25,26]. Nasal patency and releasing nasal secretion are controlled by autonomous nerve system mainly. Different peptides like vasoactive intestinal peptide (VIP), calcitonin gene related peptide (CGRP), substance P and Ach work in this innervation [27]. It is thought as mechanism of Btx-A in the treatment of AR that Btx-A causes inhibition of Ach release from cholinergic nerves in nasal mucosa and preganglionic nerves in sphenopalatine ganglion. In addition, it can cause apoptosis induction in nasal glands. As a conclusion, Btx-A can reduce parasympathetic effects in nasal cavity [5,7].

Btx-A is used in nasal cavity as topical or infiltrative. In topical application, Btx-A impregnated cotton pad or gel is used. In infiltration method, Btx-A is injected in inferior or middle turbinate and septum. While there is not any side effect in topical use; nasal dryness and epistaxis are reported in infiltration method [6–8,25]. Btx-A spreads in a 2.5–3 cm area by diffusion [28]. Two or 3 days are needed to start clinical effect of Btx-A after injection. The maximum effect is achieved in 2 weeks. Its effects decrease over time because of Ach re-synthesis and release in neuromuscular junction. Termination of effects usually takes 3–6 months [29]. Toxic dose of Btx-A is 2500–3000 U. But there is no consensus about the therapeutic dose in nasal cavity [30]. Different doses between 4–100 U have been used in different studies [5–8,31]. Unal et al. found no difference in terms of symptomatic improvement between 40 and 60 U applications in a 34-patient group without any complication or side effect. They preferred infiltration form in inferior and middle turbinates [5]. Shemshadi et al. applied 100 U Btx-A impregnated cotton pad for 30 min to 44 patients with AR and found significant difference in terms of recovering between control and Btx-A group. They reported no side effect [26]. In a 50-patient study reported by Abtahi et al., there were 2 groups that are injected 40 U Btx-A as inferior turbinate group and septum group. They found no significant difference in terms of symptomatic improvement between the two groups. They reported epistaxis in 3 patients in septum group and 1 patient in turbinate group [25].

Zhu et al. reported that 100 U equivalent gel form of Btx-A is effective for reducing AR symptoms in an animal model [8]. In an experimental study in rats made by Wen et al., 10 U Btx-A impregnated cotton pad was put for 60 min in the nose of AR created rats. They observed that this application reduces AR symptoms in rats. They also confirmed that Btx-A cause recovery in

histopathological findings like eosinophils proliferation, vascular congestion, edema and serous gland hyperplasia [7].

Turbinate hypertrophy occurs in AR patients because of different endogenous and exogenous agents. Although all mucosal surface is effected, the most effected area is inferior turbinate [32]. Inferior turbinate and infiltration method were preferred in the presented study because of their easy-accessible location and one-dose application feature. Infiltration method can be used in outpatient clinic with lower doses. Topical method needs repeated doses. For this reason, infiltration method is more cost-effective.

The effects of topical Btx-A application on histopathological findings are reported in experimental animal studies. But there is no study that investigated effects of infiltrative Btx-A application on histopathological findings before. This is the first study about this issue.

In this study; reducing of nasal discharge and histopathologic recovery were observed after Btx-A infiltration with minimal epistaxis. Because of its experimental nature, side effects like burning and dryness in the nose could not be evaluated.

5. Conclusion

Btx-A is effective in terms of reducing rhinorrhea and histopathological recovery in an animal model. It can be said that Btx-A has protective and therapeutic effect on AR.

Btx-A infiltration can be a treatment option in the patients who have treatment-resistant AR or do not want to use medication a long time and cannot use medication because of side effects with its advantages like one-dose and outpatient clinic application, reversible effect and protect normal physiology.

Conflict of interest

Authors declare that we have no commercial or conflict of interest.

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