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Research Paper

Zn/Ga – DFO iron–chelating complex attenuates the inflammatory process in a mouse model of asthma

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ABSTRACT

Background: Redox-active iron, a catalyst in the production of hydroxyl radicals via the Fenton reaction, is one of the key participants in ROS-induced tissue injury and general inflammation. According to our recent findings, an excess of tissue iron is involved in several airway-related pathologies such as nasal polyposis and asthma.
Objective: To examine the anti-inflammatory properties of a newly developed specific iron–chelating complex, Zn/Ga – DFO, in a mouse model of asthma.
Materials and methods: Asthma was induced in BALBc mice by ovalbumin, using aluminum hydroxide as an adjuvant. Mice were divided into four groups: (i) control, (ii) asthmatic and sham-treated, (iii) asthmatic treated with Zn/Ga – DFO [intra-peritoneally (i/p) and intra-nasally (i/n)], and (iv) asthmatic treated with Zn/Ga – DFO, i/n only. Lung histology and cytology were examined. Biochemical analysis of pulmonary levels of ferritin and iron-saturated ferritin was conducted.
Results: The amount of neutrophils and eosinophils in bronchoalveolar lavage fluid, goblet cell hyperplasia, mucus secretion, and peri-bronchial edema, showed markedly better values in both asthmatic-treated groups compared to the asthmatic non-treated group. The non-treated asthmatic group showed elevated ferritin levels, while in the two treated groups it returned to baseline levels. Interestingly, i/n-treatment demonstrated a more profound effect alone than in a combination with i/p injections.
Conclusion: In this mouse model of allergic asthma, Zn/Ga – DFO attenuated allergic airway inflammation. The beneficial effects of treatment were in accord with iron overload abatement in asthmatic lungs by Zn/Ga – DFO. The findings in both cellular and tissue levels supported the existence of a significant anti-inflammatory effect of Zn/Ga – DFO.

Introduction

Asthma is a chronic inflammation of the lungs in which the airways (bronchi) are reversibly narrowed. Asthma affects 7% of the population, ~300 million people worldwide. During attacks (exacerbations), the smooth muscle cells in the inflamed bronchi constrict and the airways swell, causing breathing difficulties. The frequency of acute asthmatic attacks depends on asthma severity. Acute asthma exacerbations cause 4000 deaths per year in the USA. Attacks may be prevented by avoiding triggering factors and by medical treatment. Drugs commonly used for treatment of acute attacks are inhaled β2 agonists. In severe cases, drugs are used for long-term prevention. They include inhaled corticosteroids, which may be supplemented with long-acting β2-agonists when necessary, leukotriene antagonists, which are less effective than corticosteroids but have no side effects, and monoclonal antibodies such as mepolizumab and omalizumab.

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According to several reports, asthma attacks are associated with aggravation of the inflammatory condition and with a significant increase in the production of reactive oxygen-derived species (ROS), including free radicals (FR) [1].

Iron is an essential element in all tissues and cells. However, an excess of labile iron is deleterious and causes cellular injury. This two-phase behavior is also shared by ROS – at low levels it functions as a beneficial signaling species, but at higher concentrations, specific FRs may cause damage. Labile, redox-active iron serves as a catalyst in the production of hydroxyl radicals via the Fenton reaction, and is therefore one of the key participants in ROS-induced injury and development of an inflammatory condition [2]. In a well-protected cell, 95–98% of the iron is stored in ferritin, the major iron storage and detoxifying protein, keeping labile redox-active iron at sub-micromolar levels. An increase in the concentration of cellular ferritin indicates that an initial event has occurred, during which the cellular levels of redox-active iron had increased, but were subsequently detoxified by the additional ferritin [3,4]. Various pathologic conditions, including asthma, have been proposed as being associated with such an increase in the cellular levels of iron and ferritin, presumably in response to a rise in ROS production [5]. This increase may stem as well from asthma-induced up-regulation of divalent metal transporter-1 (DMT1), transferring Fe^{2+} from extracellular matrix into the cell [6]. Rise in iron concentration leads to enhanced production of cellular ferritin to store this iron in a catalytically less reactive state.

Asthma is known to be associated with a severe form of another human pathology, nasal polyposis (NP) [7]. The role of labile iron redistribution in the development of the inflammatory process and in the pathogenesis of NP was suggested in our recent report [8]. We detected a significant increase in ferritin and ferritin-bound iron in nasal polyps from asthmatic patients. This increase was accompanied by an up-regulation of the protein carbonyls content (PCC), an oxidative stress marker, and a decrease in the concentrations of methionine-centered redox cycle proteins, which usually protect the cell against ROS-induced injury and, therefore, against inflammation.

In 1991, the idea of a "push-and-pull" mechanism to scavenge redox-active transition metals was suggested by Chevion [9]. He proposed that metal chelators, such as desferrioxamine (DFO), are able to tightly bind redox active metals such as iron and copper, thus preventing their participation in the ROS-producing Fenton reaction, diminishing therefore the level of oxidative damage [10] and even reducing production of pro-inflammatory cytokines [11]. While DFO is bound with zinc, gallium, or other redox-inactive metals, these metals are easily released in the presence of iron due to a higher DFO affinity to iron, being "pushed away", thus leading to iron displacement, and diverting the site of FR attack. The usage of zinc–DFO and gallium–DFO complexes has additional advantages, since anti-inflammatory, and even specifically anti-asthmatic, effect of these metals has been reported [12–16].

DFO by itself is a relatively large, randomly oriented non-polar molecule, that does not easily penetrate cells, and provides only a minimal protection against FR damage [17]. However, when complexed with gallium or with zinc, it assumes a well-defined compact structure, likely enhancing its ability to infiltrate cell membranes. The pathophysiological effects (e.g. chemical or alkali burns and cardiac ischemia-reperfusion injury) of these complexes were tested in various animal models [9,17–21].

Animal models mimicking the pathophysiology of human asthma are important tools for studying the mechanisms of AllerGen-induced asthma, airway hyper-responsiveness (AHR), airway inflammation, and reversible airway obstruction (National Heart, Lung, and Blood Institute. Update 2009. Global Initiative for Asthma Global strategy for asthma management and prevention. NHLBI/WHO workshop report). Several reports [22–24] have used AllerGen-sensitized mice to describe the roles that cells and cytokines play in the development of AHR, demonstrating the usefulness and acceptability of this animal model. The BALB/c strain of mice, known to be highly susceptible to asthma induction protocols [25–27], serve as the gold standard for studying inflammatory and bronchial hyper-reactive airways.

The objective of the current study was to evaluate the effect of Zn/Ga – DFO complexes as specific iron chelators in the treatment of asthma.

Materials and methods

Complex preparation

To prepare zinc-/gallium-complex with desferrioxamine, a 10 millimoles solution of desferrioxamine mesylate was mixed with equal volume of $ZnCl_2$ or $GaCl_3$ 10 millimoles solution and titrated to pH 7.4. To form the complex, the mixture was heated to 45 °C for 30 min. The metal:DFO ratio in the complex was 1.0:1.0. A 3:1 mixture of Zn – DFO and Ga – DFO was applied.

Model description

All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Technion – Israel Institute of Technology, Haifa, conforming to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH, Publication 85-23, revised 1996). The experiments were performed using an animal model of asthma induced by ovalbumin (OVA) sensitization [28]. BALB/c female 8 w. o. mice, purchased from Harlan, Israel, were divided into 4 groups ($n=8$): (1) control; (2) asthmatic, sham-treated; (3) asthmatic, treated with Zn/Ga – DFO complex intra-peritoneally (i/p) and intra-nasally (i/n); (4) asthmatic treated with Zn/Ga – DFO complex, i/n only. The dose and pattern of administration were based on previous experiments performed by Chevion [9,18,29]. The animals were sensitized to ovalbumin by intra-peritoneal 100 μl injection (10 μg ovalbumin and 3 mg $Al(OH)_3$ in 0.9% saline) on days 0, 7, and 14. The mice were further sensitized with intra-nasal instillations of OVA (50 μl) on days 17, 19, 20, and 23. Group 3 received prophylactic treatment by 2 i/p injections (100 μl each; 1 mg of Zn/Ga – DFO complex per kg body weight in saline buffer) 5 days and 1 day before the first sensitization. Subsequently, the i/p injections containing only 1/3 of the dose (0.3 mg/kg) were given one day before and one day after the OVA sensitization, while a dose of 1 mg/kg weight was given on the day of sensitization. From day 15, the complex was given i/n.

Group 4 received 5 mg/kg of Zn/Ga – DFO i/n only, according to the same administration pattern. Animals in the control non-asthmatic group received saline injections and instillations, using the same regime. At the end of the experiments, Day 24, the animals were sacrificed with a ketamine/xylazine mixture.

Biochemical parameters

The lung samples were homogenized in lysis buffer using a Teflon homogenizer. Ferritin concentration was quantified using an indirect 'sandwich' ELISA assay, accordingly to the procedure developed previously in our laboratory [30]. Ferritin was immunoprecipitated and ferritin-bound iron was measured as previously described [31].

Histology parameters

BAL using 1 ml of saline solution was performed immediately after sacrifice. BAL fluid on cytospin slides was fixed and stained with Diff-Quick, and counted under a light microscope. Following lavage, the lungs were excised, filled with a 4% paraformaldehyde solution, and sliced longitudinally into 3 parts. The middle third was embedded in paraffin, randomly sliced, and stained with eosin–hematoxylin. The intensity of the peribronchial and perivascular cellular infiltration and mucous content was assessed ($n=5$ per group).

Periodic Acid Schiff (PAS) staining was used to assess epithelial cells metaplasia, and Masson's trichrome staining was used to evaluate the presence of fibrous connective tissue.

Statistical analysis

The data was analyzed using repeated one-way ANOVA followed by the Scheffe post-hoc test for multiple comparisons (with $\alpha=0.05$). Differences between mean values with $p \leq 0.05$ were considered statistically significant.

Results

Thirty two mice were studied, four groups of 8 mice.

The concentrations of ferritin in the lungs are shown in Fig. 1A. The baseline ferritin concentration, observed in the control lungs, was 0.17 ± 0.02 μg ferritin/mg protein. A significant increase was found in asthmatic (non-treated) lungs, at 0.45 ± 0.06 μg/mg protein. In i/n treated lungs, a value of 0.28 ± 0.04 μg ferritin/mg protein was observed, and in the lungs treated by both i/p and i/n, ferritin concentration was down-regulated to a level only slightly above the control group.

Although the levels of ferritin saturation with iron (Fig. 1B) in the lungs of asthmatic and control mice were similar, the total amount of ferritin-bound iron (FBI) was 2.7 times higher in asthmatic mice lungs, due to a higher ferritin concentration (Fig. 1A). Treatment with Zn/Ga – DFO, either i/p + i/n, or i/n only, decreased both the general amount of ferritin and the level of iron saturation. There were no significant differences in FBI between the treated groups.

The amounts of eosinophils and neutrophils in BAL fluid are shown in Fig. 2. The eosinophil count (Fig. 2A) from the control group was the lowest at $2.0 \pm 0.5 \times 10^4$ cells/ml, but asthma caused ~5.5 fold increase ($p < 0.05$). No statistically significant differences were found between asthmatic mice lungs and lungs from the i/p + i/n group. However, sub-baseline levels were found in the i/n only group.

Neutrophil count (Fig. 2B) in control mice was $6.8 \pm 2.3 \times 10^4$ cells/ml. In non-treated asthmatic mice, a 6.3-fold increase was detected ($p < 0.05$). In the asthmatic-treated group 3 (Zn/Ga – DFO, i/p + i/n), the value decreased to $29.8 \pm 4.3 \times 10^4$ cells/ml, but was still significantly above the control value ($p < 0.05$). In i/n-only treated mice, neutrophils concentration decreased to slightly below baseline, $2.0 \pm 0.4 \times 10^4$ cells/ml, which was significantly different ($p < 0.05$) from the values found in asthmatic sham-treated and in the i/p + i/n-treated asthmatic mice.

Comparing mucous content values (Table 1), we found that both methods of treatment were able to reduce mucous levels to the baseline values.

Histological parameters are shown in Fig. 3. The histological score was based on integer values scaled from 0 to 3. In general, the different experimental groups showed congruent results for peri-bronchial staining for fibrous connective tissue (C). Similar to the previous results, the asthmatic group received the highest average score at ~2.5, while the control level was 0. Both modes of treatment succeeded in decreasing the asthma-associated parameters by at least 1.5-fold, however, the i/n treatment showed a more profound effect than the i/p + i/n treatment. Representative slides of PAS staining demonstrating the effect of Zn/Ga – DFO on epithelial cell metaplasia are shown in Fig. 4.

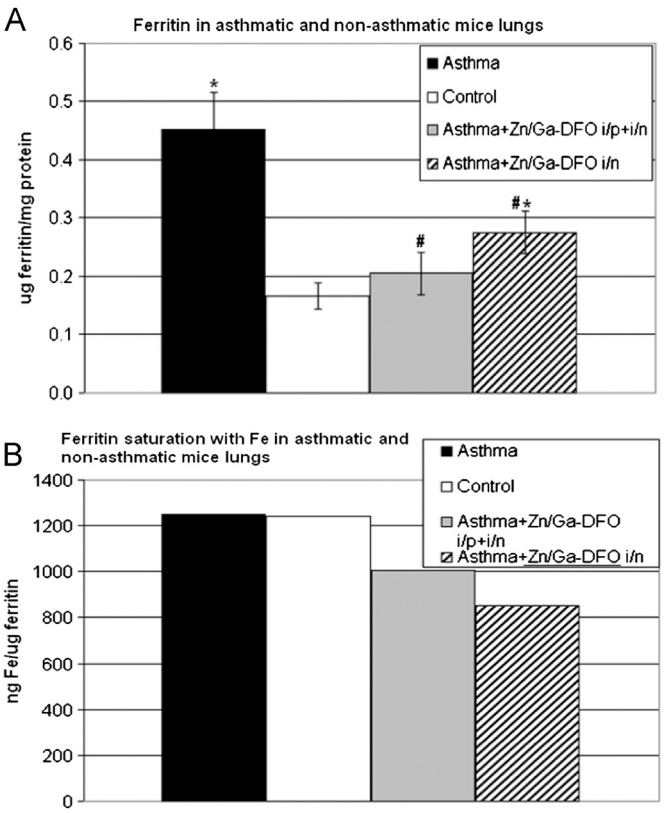

Fig. 1. The concentration of ferritin (A) and Fe-saturated ferritin (B) in mice lungs. Ferritin concentration was quantified using an indirect 'sandwich' ELISA assay in accordance with a procedure developed previously in our laboratory [30]. ELISA 96-well microplates were pre-coated with goat anti-rat L-ferritin antibody. Rabbit anti-rat H-ferritin was used as the secondary antibody. Plates were treated with goat anti-rabbit IgG conjugated with β-galactosidase. Chlorophenol Red-β-D-Galactopyranoside was then added and the plates were analyzed using a microplate reader with test (570 nm) and reference (630 nm) filters. Ferritin-bound iron was measured as follows. In order to reach a concentration of 2 mg ferritin/1 ml, several samples from each group were pooled together. Ferritin was immune-precipitated using a mixture of anti-H and anti-L ferritin antibodies, developed in our lab. The precipitate was dissolved in nitric acid, and iron content was determined spectrophotometrically with batho-phenanthroline bi-sulfonate, using 535 nm filters. Means ± SE are shown. * Denotes $p < 0.05$ *vs.* the control; # denotes $p < 0.05$ *vs.* the asthmatic non-treated group.

Discussion

The present study focused on the role of iron and iron-catalyzed oxidative injury in the asthmatic inflammatory process. Recently, we showed that iron re-distribution plays an important role in the NP inflammatory pathway, and that this effect was exaggerated in asthmatics [8]. In the current study, we sequestrated iron using Zn/Ga – DFO, a specific iron chelator developed in our laboratory, and tested its effects on several asthma-associated parameters using a classic murine OVA model.

Maintenance of iron homeostasis is of utmost importance to the physiology and pathophysiology of the respiratory system. Local iron deficiency can impair the growth and proliferation of cells responsible for inflammatory response and tissue repair [32,33]. Cellular iron homeostasis is maintained through post-transcriptional regulation of

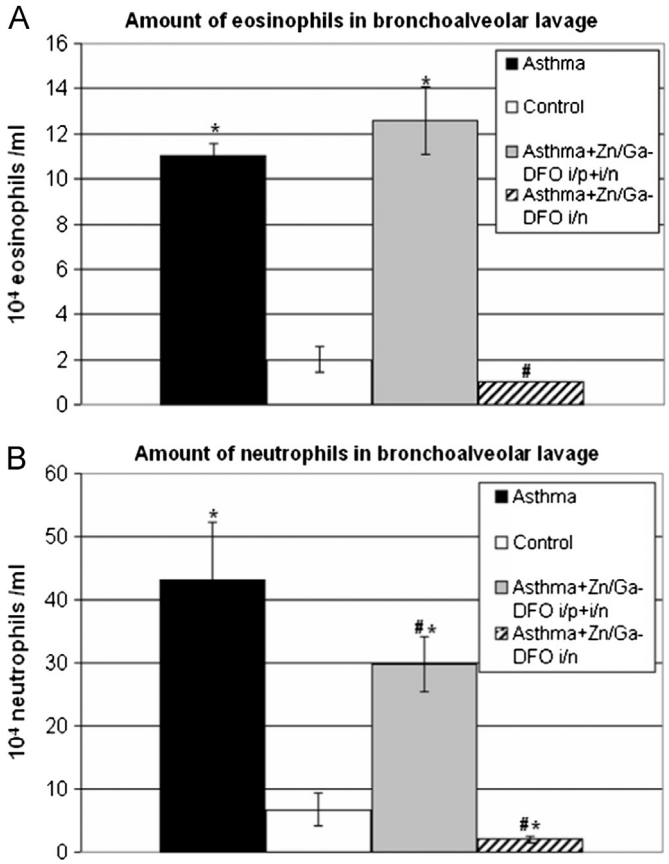

Fig. 2. The amount of eosinophils (A) and neutrophils (B) in mice BAL fluid. To estimate the anti-inflammatory effect of the treatment provided, the amounts of eosinophils and neutrophils in bronchoalveolar lavages (BAL) were measured. BAL using 1 ml of a buffered saline solution was performed immediately after the ketamine/xylazine injection. BAL fluid on cytospin slides was fixed and stained with diff-quick. Differential cell counts were obtained under a light microscope. Mean ± SE is shown. * Denotes $p < 0.05$ *vs.* the control; # denotes $p < 0.05$ *vs.* the asthmatic sham-treated group.

Table 1
Mucous content in mice lungs.

Group	Mucous content value
Asthma	0.8 ± 0.2*
Control	0
Asthma+Zn/Ga−DFO i/n	0
Asthma + Zn/Ga–DFO i/p+i/n	0

Average ± ME is shown.
* Denotes $p < 0.05$ *vs.* the control.

ferritin and transferrin receptor (TfR) via iron regulatory proteins (IRP). In the mRNA of ferritin and TfR, there are iron-responsive elements (IRE) that can bind or release the IRPs, thereby blocking or enabling translation and protein synthesis [34]. Inflammatory mediators, such as hydrogen peroxide and nitric oxide, may also regulate IRP activity in order to increase ferritin and TfR synthesis during inflammatory processes. In fact, ferritin accumulation and inflammation have been linked by several published reports [35,36]. Understanding the relationship between iron homeostasis and inflammation is of great importance, especially since the respiratory tract is open to the external environment and constantly exposed to potentially inflammatory stimuli [37]. The therapeutic effect of DFO is based on a chelation of labile, redox-active iron, directly associated with an inflammatory process.

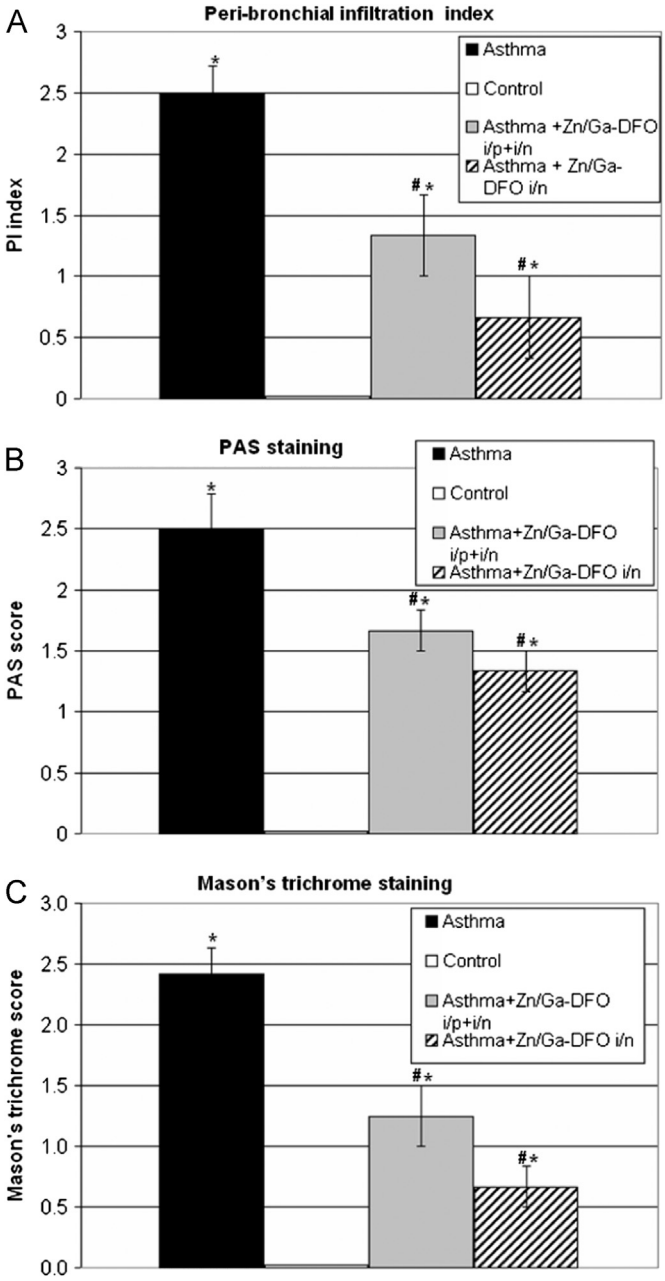

Fig. 3. Histology scores of mice lungs from the experimental groups: peribroncheal infiltrate (A), PAS (B) and Mason's trichrome (C) staining. Following lung lavage, the lungs were excised and filled paraformaldehyde and sliced longitudinally into 3 parts. The middle third was embedded in paraffin, randomly sliced, and stained with eosin-hematoxylin. The intensity of the peribronchial and perivascular cellular infiltration was assessed semi-quantitatively by light microscopy on a 0–3 scale as follows: 0=no, or practically no inflammatory cells; 1=a narrow rim of inflammatory cells surrounding most of the bronchioles/blood vessels, best visualized by high power filed; 2=a rim of inflammatory cells 3–4 cells thick, surrounding most of the bronchioles/blood vessels; and 3=a prominent rim of inflammatory cells, 5 or more cells thick, surrounding most of the bronchioles/blood vessels. Periodic acid Schiff (PAS) staining was used to assess metaplasia of epithelial cells, and Masson's trichrome staining was used to evaluate the presence of fibrous connective tissue. Both were scored as follows: 0 – none, 1 – mild, 2 – moderate, 3 – significant [47]. Histological assessment was performed by an independent pathologist, blinded to the study groups. Means ± SE is shown. * Denotes $p < 0.05$ *vs.* the control; # denotes $p < 0.05$ *vs.* the asthmatic non-treated group.

Treatment with Zn/Ga−DFO complexes via both administration methods decreased the level of ferritin and the level of FBI in asthmatic mice lungs (Fig. 1). However, the treatment by intranasal

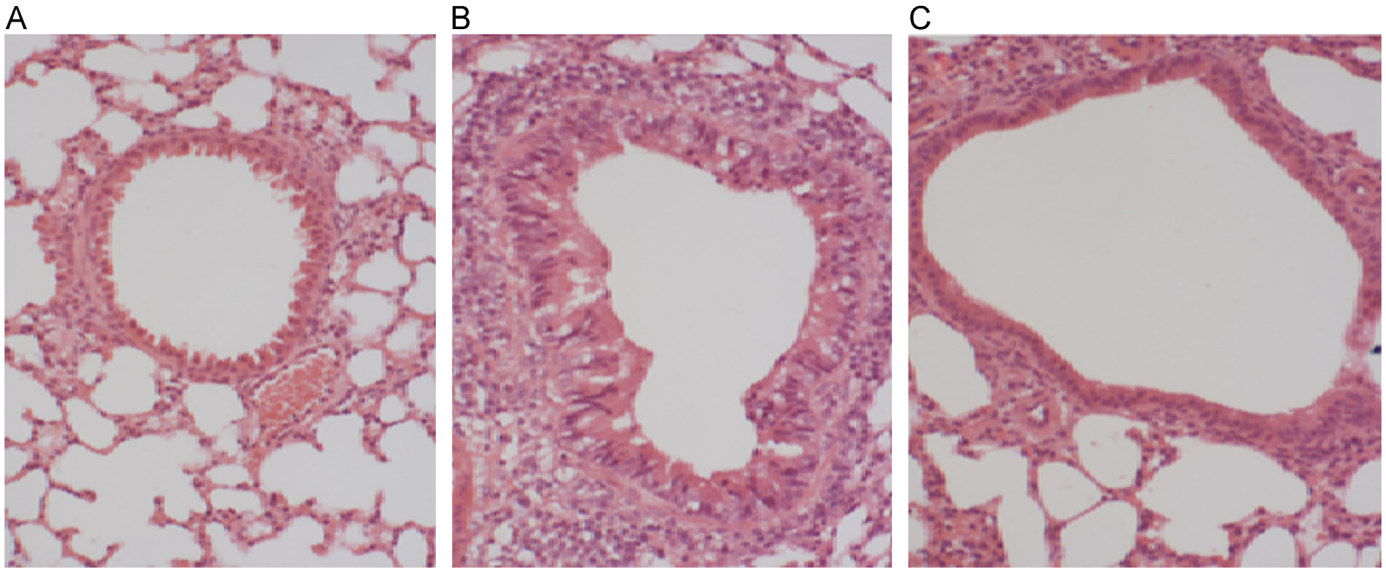

Fig. 4. Representative slides of PAS-stained lungs: (A) control; (B) asthmatic; (C) Zn/Ga – DFO i/p + i/n-treated.

instillations alone demonstrated a more profound therapeutic effect. Since the allergenic effect of OVA extends through the whole organism [38], we expected a general inflammation-associated increase in the concentration of labile iron. Thus, only a part of Zn/Ga – DFO injected i/p reached the site of inflammation in active form without iron ion bound. On the other hand, while the complex, from the beginning of the treatment, was administered by intranasal instillation, being applied almost directly on the site of inflammation, the pathogenic local iron accumulation in the lungs was immediately reduced, and therapeutic effect was significantly improved.

Kruzel et al. showed that lactoferrin (LTF), a pleiotropic 80-kDa glycoprotein with iron-binding properties, exhibited a potent anti-allergic effect [39]. Bournazou et al. showed that LTF acted as a powerful specific inhibitor of eosinophil migration [40]. Our results (Fig. 2) corroborate these findings. However, only local treatment via intra-nasal instillations decreased the level of eosinophils in the BAL of asthmatic mice lungs.

Neutrophil levels in BAL fluid was reduced by both methods of administration. Consistently with our previous observations, intra-nasal instillations demonstrated a much higher efficacy in reducing this parameter compared to systemic treatment by i/p injections. Both methods of treatment for clearing asthma-induced respiratory mucous were successful, eliminating it completely.

The same trend was observed in all parameters of the three histology scores (Fig. 3). A pivotal role of ROS in asthma-induced lung remodeling was reported [41]. Chelation of labile iron, involved in ROS formation, by Zn/Ga – DFO complex did not completely abolish asthma-induced lung remodeling, but reduced it significantly, and, similar to the results observed in the BAL experiments, intra-nasal instillations were much more effective than i/p injections. In contrast to iron accumulation, tissue remodeling and white blood cell infiltration are local processes, and direct application of the drug *in situ* is markedly more effective than systemic treatment. Comparing the results of two assays employing an OVA-induced murine model of asthma, one using the Zn/Ga – DFO complex, a potentially novel anti-asthmatic drug, and one using 2-Methoxyestradiol (ME), a steroidal anti-asthmatic compound, one may conclude that the novel complex exerts an equivalent, if not a better effect as ME [42]. However, in the current study, animals were subjected to a prophylactic treatment, while ME was administered after the onset of asthma.

In addition to their effects together with DFO, zinc and gallium by themselves bear anti-oxidant and anti-inflammatory potentials [12–16], with reports that zinc possibly possess direct anti-asthmatic effects as well [43,44]. Thus, the newly suggested Zn/Ga – DFO complex probably acts via the combination of three different mechanisms – anti-oxidative, anti-inflammatory, and iron–chelating – simultaneously. These findings are in accord with a line of previous publications, showing a beneficial effect of Zn/Ga – DFO complex on several organs, exposed to different kinds of stress, such as heart and retina under ischemia-reperfusion conditions, and chemically injured cornea or lens under hyperbaric oxygen conditions. In these applications, the complex has been demonstrated to reduce the levels of malondialdehyde and DHBA (the indicators of lipid peroxidation and hydroxyl radical formation, respectively), and generally increased a systemic antioxidant status [20,21,29,45].

According to the results by Obolensky et al. [46], the components of the complex, Zn and DFO, when administered alone, showed a lesser, partial effect, while the conclusion about the synergy, existing between zinc and DFO in the Zn/Ga – DFO complex can be drawn.

We conclude that Zn/Ga – DFO complex has a significant potential as an anti-asthmatic drug. However, several assays are required in order to clarify its mechanism of action, to study its effects in humans, and to investigate its systemic effects.

Acknowledgments

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