Zinc-depletion associates with tissue eosinophilia and collagen depletion in chronic rhinosinusitis*

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Abstract

Background: Zinc plays an important role in many biological processes. Reduced zinc levels have been found in chronic rhinosinusitis (CRS) patients, however, its role in the pathophysiology of this disease remains unknown. This study examined zinc levels in the serum, mucus and tissue from CRS patients in relation to collagen content and eosinophil infiltration. The effect of zinc depletion on inflammatory cytokine production and collagen synthesis was assessed in vitro.

Methodology: Zinc levels were determined in serum, mucus and tissue from controls, CRS with (CRSwNP) and without nasal polyps (CRSsNP) patients. Tissue zinc levels, collagen and inflammatory cell infiltration was examined using zinquin assays, immunofluorescence and histology on Tissue Micro Arrays. Cytokine expression and collagen synthesis was evaluated in zinc depleted primary human nasal epithelial cells (HNECs) and primary fibroblasts.

Results: CRSwNP patients showed reduced tissue zinc levels in correlation with a reduction in collagen content, and increased eosinophil numbers. Zinc depletion of HNECs and fibroblasts induced the production of pro-inflammatory cytokines and MUC5AC and reduced collagen secretion.

Conclusions: These results suggest mucosal zinc depletion associates with tissue eosinophilia and collagen depletion in CRSwNP and induces pro-inflammatory cytokine expression and reduction of collagen synthesis in vitro.

Key words: human nasal epithelial cells, nasal polyps, Zinquin

Introduction

The pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP) has not yet been fully elucidated and several factors have been proposed to play a role [1,2]. CRSwNP is characterized by an accumulation of inflammatory cells, stromal edema and tissue remodeling. Up to 70% of CRSwNP patients present with high eosinophil infiltration within the mucosa and nasal polyps and those patients often have serum eosinophilia, atopy, extensive disease and poor prognosis compared to non-eosinophilic CRS patients [3,4]. Eosinophilic CRS is characterised by a Th2-skewed immune response with infiltration of Th2 cells and Type 2 Innate Lymphoid Cells that are thought to be involved in the initiation and coordination of those responses [5,6]. CRSwNP patients have also been shown to have decreased collagen levels [7,8] in association with reduced transforming growth factor-β (TGF-β) signalling and defective tissue repair mechanisms with loose connective tissue and edema formation [9]. Tissue remodelling and repair and the balance of collagen synthesis and degradation as well as multiple aspects of innate and adaptive immunity are critically dependent on adequate zinc levels [10].

Zinc is an essential micronutrient and a vital cofactor for the

function of more than 10% of proteins encoded by the human genome [11]. Consequently, zinc depletion affects multiple organs and systems and causes impaired immune function and compromised wound healing. CRSwNP patients have been shown to present with lower mucosal zinc levels in association with a reduced expression of the tight junction protein Zonula Occludens-1 (ZO-1) compared to controls [12]. Interestingly, primary human nasal epithelial cells (HNECs) grown in a zinc depleted medium formed leaky mucosal barriers implicating zinc homeostasis also in establishing functional mucosal barriers [12]. In the lower airway, decreased serum zinc levels have been shown to be associated with severe asthma [13,14]. Zinc depletion is associated with a Th2-dominant immune response in lower airway epithelium with infiltration of eosinophils [15].
Despite these established links between zinc depletion and tissue eosinophilia, immune dysfunction and impaired wound remodeling processes, the significance of decreased zinc levels in the context of CRSwNP and how this relates to collagen levels and tissue eosinophilia still remains to be clarified. This study used patient-derived tissue samples to determine zinc levels in relation to tissue eosinophilia and collagen levels in the context of CRS. In addition, primary human nasal epithelial cells and primary nasal fibroblasts were used to assess the effect of zinc depletion on the proliferation and production of cytokines and collagen.

Material and methods

Patients and tissue collection

The study was approved by The Queen Elizabeth Hospital Human Research Ethics Committee (reference HREC/15/TQEH/132) and written informed consent was obtained from donors prior to tissue, serum, mucus or cell collection. Study participants included those undergoing endoscopic sinus surgery (ESS) and were defined as controls, CRSsNP or CRSwNP. Control patients were undergoing ESS for skull base tumor resection, and were only included in the absence of radiographic or endoscopic evidence of sinusitis. CRS patients were divided into CRSsNP and CRSwNP depending on the absence or presence of nasal polyps respectively, as defined by the European Position Paper [16]. A retrospective cohort study design was used for the immunofluorescence analysis. Ethmoidal mucosa was used in control and CRSsNP patients and nasal polyps were used for CRSwNP patients. Exclusion criteria included immunosuppression and treatments with oral antibiotics or corticosteroids in the week prior to study inclusion.

Primary human nasal epithelial cell cultures

Primary human nasal epithelial cells were obtained from the inferior turbinate mucosa of healthy donors using nasal brushings in a method as previously described [17-19]. The cells were suspended in Bronchial Epithelial Growth Medium (BEGM, CC-3170, Lonza, Walkersville, MD, USA), supplemented with 2% Ultroser G (Pall Corporation, Port Washington, NY, USA). The cell suspension was depleted of monocytes using anti-CD68 antibody (Dako, Glostrup, Denmark) coated cell culture dishes. HNECs were expanded in routine cell culture conditions (37°C, humidified, 5% CO_2) in collagen-coated flasks (Thermo Scientific, Walthman, MA, USA). Qualified cytologists (IMVS Cytology Department, The Queen Elizabeth Hospital, Adelaide, Australia) confirmed the cells were of epithelial lineage by reactivity to PAN-cytokeratin and CD45 antibodies, and by morphological examination with Diff-Quik analysis. HNECs were used at passage 2.

Primary human nasal fibroblast cultures

Fibroblasts were isolated from human nasal tissue as described [20]. Briefly, sinonasal tissue was dissected into 1x1 mm^2 pieces and incubated for 2–4 weeks in Dulbecco modified Eagle medium (DMEM; Gibco, Invitrogen, Melbourne, Australia). The tissue fragments were removed when cells had grown to form a confluent monolayer. The mixed cell population was trypsinized, pelleted, and resuspended in phosphate buffered saline (PBS) before pure fibroblast cells were isolated by immunomagnetic bead separation (Epithelial Enrich Dynabeads; Invitrogen, NY, USA) to remove epithelial cells. Supernatants containing fibroblasts were transferred to a T25 tissue culture flask (Nunc, Roskilde, Denmark) and the tube containing the remaining beads discarded.

Zinc depleted media

In two separate 50 ml falcon tubes, 5 grams of analytical grade Chelex 100 chelating ion-exchange resin (C7901, Sigma, Saint Louis, MO, USA) was washed 2 × with Milli-Q water for 10 minutes. After washing, 50 ml of BEGM or DMEM was added into the first tube and incubated for 2 hours on a rotator at room temperature (RT). Then the media was transferred from the first tube into the second tube and incubated overnight on a rotator at 4°C. Next day, the media was collected and the Chelex beads were discarded. The depleted molecules except zinc were added to the media ($MgSO_4$=2.4mg, $MgCl_2$=4.28mg, $CaCl_2$=22.19mg, $Fe(NO_3)_3$=0.00014mg, $FeSO_4$=0.022mg). The pH of the media was determined, and the media was sent to SA Pathology Australia to detect any zinc elements.

Viability assay

HNECs or fibroblasts were treated with zinc depleted and non-depleted media for 48 hours respectively at 37°C in 5% CO_2. Then the amount of lactate dehydrogenase (LDH) in the medium was measured with a Cytotoxicity Detection Kit (Promega, Madison, WI, USA). Briefly, 50 µl of the media from each well was transferred to a new plate, and 50 µl of LDH reagent was added to the supernatant and incubated for 30 minutes in the dark at 37°C. The Optical Density (OD) was measured at 490 nm

on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

Proliferation assay

HNECs were established at 0.5 × 106 cells/ml in a 24-well plate and incubated overnight at 37°C in 5% CO_2 to allow adherence. Then cells were treated with zinc depleted and non-depleted media for 48 hours at 37°C in 5% CO_2. Cells were harvested by trypsinisation then fixed with 3 ml ice-cold 70% ethanol at -20°C overnight. The cell pellet was resuspended in 1 ml of mixture solution (20 µg/ml of propidium iodide (PI) and 200 µg/ml of RNase (R-5503, Sigma, Saint Louis, MO, USA) in 0.1% Triton X-100 in PBS) and incubated at RT in the dark for 30 minutes. Samples were analyzed using a BD FACSCanto™ II flow cytometer.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants were collected from HNECs or fibroblasts after incubation with zinc depleted and non-depleted media. Total Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), and collagen I protein levels were estimated with an ELISA kit using anti-human IL-6 antibody (BD Biosciences, Franklin Lakes, NJ, USA), anti-human IL-8 antibody (BD Biosciences, San Diego, CA, USA), anti-human IL-10 (Invitrogen, CA, USA), and procollagen type I C-peptide (Takara Bio Inc, Otsu, Japan), respectively, according to the manufacturer's instructions. All measurements were performed in duplicate using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). The tissue sample concentration was calculated from a standard curve and corrected for protein concentration. The results were standardized with the viable cell number.

RNA extraction, reverse transcription and qPCR

Total RNA was extracted from RNAlater stored samples using the Qiagen RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions followed by DNAse treatment with RNase-Free DNAse set (Qiagen). Extracted RNA was quantified using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Franklin, MA, USA). RNA was reverse transcribed into cDNA using Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany) with a MyCycler Thermal Cycler (Bio-Rad Laboratories Inc., Gladesville, Australia). The resulting cDNA was subjected to qPCR with TAQman primer/probe sets for each target gene, Taqman Universal Master Mix II (Thermo Fisher Scientific, Scoresby, Australia) and nuclease-free water. The average threshold cycle (Ct) was determined from three independent experiments and the level of gene expression relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined with the comparative CT method. Taqman Gene Assays used for gene expression analysis were: Hs00894040_m1 (MUC2), Hs01370716_m1 (MUC5AC), Hs00164004_m1 (COL1A1), and Hs02758991 (GAPDH).

Tissue microarray

Archival formalin-fixed paraffin-embedded sinus tissue blocks collected from patients who underwent sinonasal surgery were used to construct the tissue microarray (TMA) for subsequent analysis by immunofluorescence. The analyses were conducted with the approval of the local Human Research Ethics Committee. Prior to constructing each TMA, a 4 µm section was sliced from each tissue block and stained using Haematoxylin and Eosin (H&E) to guide the location of tissue cores for punching. TMAs were manually constructed using a 2 mm disposable biopsy punch (Kai medical, Kai Europe GmbH, Solingen, Germany) and Quick-RayTM paraffin recipient block 2 mm × 60 wells (IHC World, Woodstock, MD, USA). For the TMA slides, tissue cores were punched from representative non-CRS controls, CRSsNP, CRSwNPs. Mucosa was used for the non-CRS controls and CRSsNP patients and polyp tissue was used for the CRSwNP patients. Patients were categorised as CRSsNP or CRSwNP, according to the European Position Paper [16]. Non-CRS controls were patients that underwent pituitary tumor resection without evidence of sinus disease.

Mucus zinc concentration

Polyurethane foam (5 x 25 mm) was placed between the inferior turbinate and nasal septum for 10 minutes in patients prior to surgery. The sponge was then stored in microcentrifuge tubes with Spin-X inserts (Corning, New York, USA) at 4 °C for transfer. Nasal mucus was extracted from the sponge by centrifugation at 15,000 g for 10 mins. Tubes of nasal mucus samples were frozen at -80 °C until required for analysis. 100 µL of mucus was measured for zinc concentrations using an inductively coupled plasma mass spectrometer (ICP-MS) (Elan DRC; Perkin Elmer, MA, USA).

Serum zinc concentration

Serum was collected from 20 patients (5 controls, 10 CRSsNP, and 5 CRSwNP) that were included in the TMA. 100 µL of serum was measured for zinc concentrations using ICP-MS.

Immunofluorescence

Cells were fixed with 2.5% formalin in PBS for 10 minutes, and then the cells were rinsed with Tris-Buffered Saline-0.5% Tween (TBST) four times, permeabilized with 1% SDS in PBS, and blocked with Serum Free Blocker (SFB, Dako, Glostrup, Denmark) for 60 minutes, at RT. The sections were rinsed in TBST, and after the third wash, 25 µmol/L of zinquin ethylester (Sigma-Aldrich, St. Louis, MO, USA) was added. Slides were washed, mounted with fluorescent mounting medium (SFB, Dako, Glostrup, Denmark) and visualized by using a LSM700 Confocal Laser Scanning Microscope (Zeiss Microscopy, Oberkochen, Germany).
For immunofluorescence of TMAs, samples were cut in 4 µm sections from the TMA block, rehydrated and antigen retrieval was

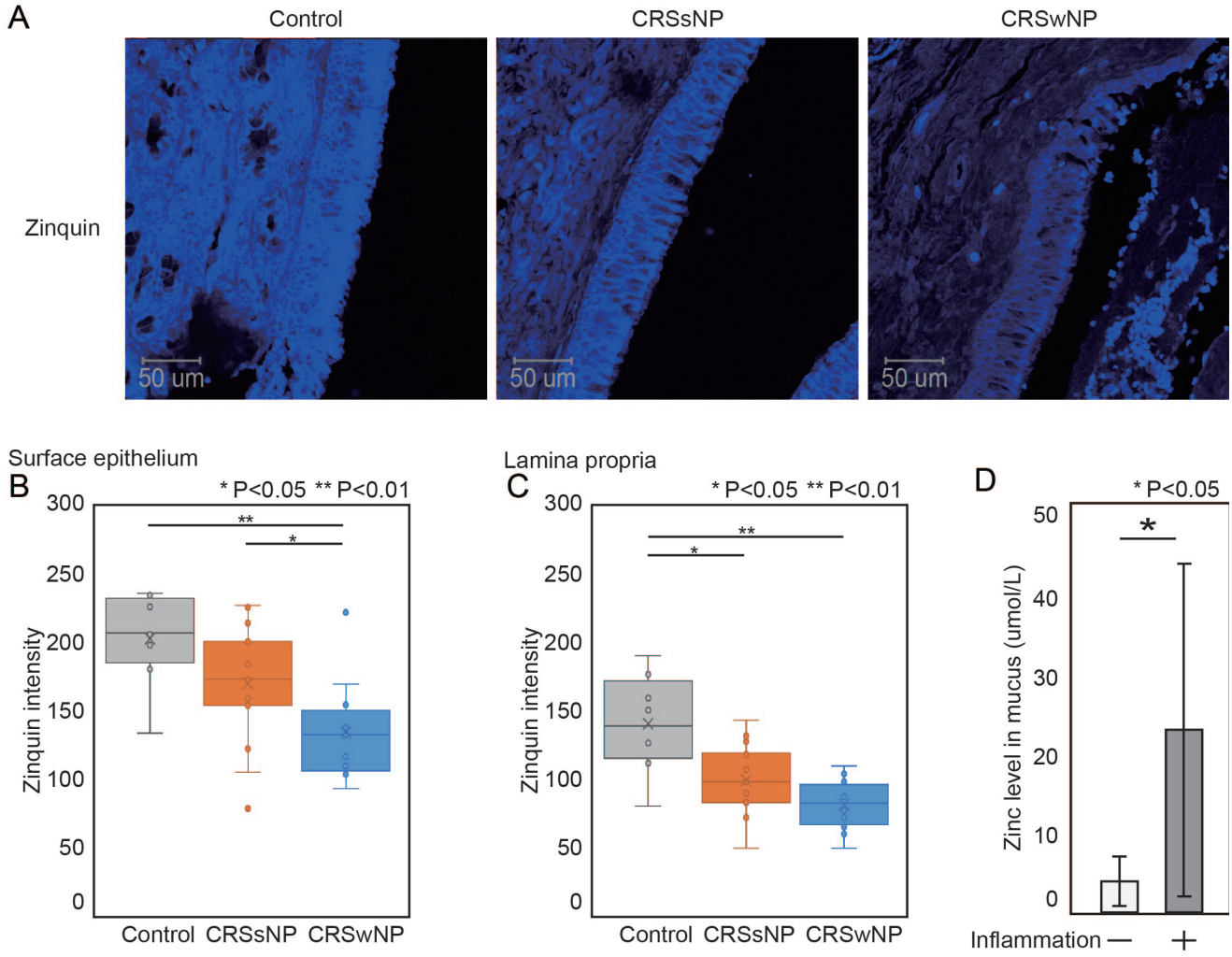

Figure 1. Labile zinc levels are decreased in the surface epithelium and lamina propria in CRSwNP. A) Immunofluorescence of representative control, CRSsNP and CRSwNP patients using zinquin assays (blue staining). B and C) Zinquin fluorescence intensity between the different patient groups was compared in the surface epithelium (B) and the lamina propria (C) in nasal mucosa. D) Comparison in zinc level in mucus from inflammatory site with that from non-inflammatory site. *p<0.05, Wilcoxon test.

performed by submerging the slides in sodium citrate buffer (10 mM sodium citrate buffer, pH 6.8) and incubating in a 1100W microwave for 10 minutes. After cooling, sections were blocked with SFB (Dako, Glostrup, Denmark) for 60 minutes at RT, followed by incubation with zinquin ethylester as described above.

H&E and Sirius Red staining and evaluation of collagen

Specimens in the TMA were cut at a thickness of 4 µm and stained with H&E or Sirius Red. The stained slides were scanned into digital files using a NanoZoomer 2.0-HT (Hamamatsu photonics K.K., Hamamatsu, Japan). The images were reviewed using NDP.view2 (Hamamatsu photonics K.K., Hamamatsu, Japan) by 3 independent observers who were blinded to the disease state of the patient. The H&E and Sirius Red images from the corresponding blocks were synchronized. For each specimen, at least 10 areas of the lamina propria measuring 0.035 mm^2 each were randomly selected as described [17,21,22]. Eosinophil numbers, all infiltrating inflammatory cells and collagen in each area were examined. To quantify the red-stained collagen, the image of each area from tissue samples was converted to gray scale using ImageJ 1.50i (National Institutes of Health, Bethesda, MD, USA). The red-stained collagen was segmented using thresholding and the area of collagen was measured and expressed as a percentage to the whole area.

Statistical analysis

All data was expressed as mean ± Standard Deviation (SD). For the immunofluorescence analysis of mucus zinc levels, the necessary sample size was calculated to be 22 cases (7.33 for each group) assuming a study power of 0.80 and alpha 0.05, based on data from previous reports on zinc level in nasal mucosa [12]. The intensity of zinquin and eosinophil count was compared

Table 1. Patient demographics in this study.

	Control (n=8)	CRSsNP (n=15)	CRSwNP (n=13)
Age	48.32±14.63	47.30±14.72	50.98±16.70
Gender (male/female)	6/2	8/7	7/6
Asthma (%)*	0 (0%)	2 (13.3%)	10 (76.9%)
Previous sinus surgery (%)	n/a	6 (40.0%)	11 (84.6%)
Zinc intensity			
Surface epithelium	207.16± 33.32	172.35± 43.33	134.57± 35.75
Lamina propria	139.61± 35.38	96.58± 25.48	77.66± 18.21
Eosinophil / HPF	18.39± 34.16	34.50± 57.54	455.60± 295.76
All infiltrating cells /HPF	390.70± 239.94	1445.81± 625.12	2098.78± 893.60

CRSsNP: chronic rhinosinusitis without nasal polyps, CRSwNP: chronic rhinosinusitis with nasal polyps, HPF: high power field.

with Kruskal-Wallis test. Cell population, cell viability, collagen I mRNA expression, collagen expression, and cytokine expression were compared with a two-tailed t-test. When 3 or more groups were compared, one-way Analysis Of Variance (ANOVA) followed by Tukey's test was used to analyze differences among the groups. The Pearson correlation test was used to determine the correlation between zinquin, collagen, eosinophil count, and all inflammatory cell count. P-values of <0.05 were considered statistically significant. All the analyses were performed by using JMP® 11 software (SAS Institute Inc., Cary, NC, USA).

Results

Labile zinc levels are reduced in the mucosa of CRSwNP patients

First, we examined labile zinc levels within the mucosa using zinquin assays on a TMA slide that included tissue cores from 8 controls, 15 CRSsNP, and 13 CRSwNP (Figure 1A). These included 21 male, 15 female patients aged 33-75 years old with an equal distribution of age and gender across the groups and with 12/28 CRS patients being asthmatic (Table 1). Zinquin fluorescence intensity was measured at different areas within the mucosa (surface epithelium and lamina propria) and compared amongst patient groups. Zinquin intensity in CRSwNP tissue was significantly decreased in both surface epithelium (P=0.0023 to controls and P=0.0233 to CRSsNP, Figure 1B) and lamina propria (P=0.0014 to controls, Figure 1C). Zinquin intensity in CRSsNP tissue was also significantly decreased in the lamina propria compared to controls (P=0.0185, Figure 1C). Labile zinc was mainly observed within the surface epithelium and epithelial cells of glands and ducts (Supplementary Figure 1).

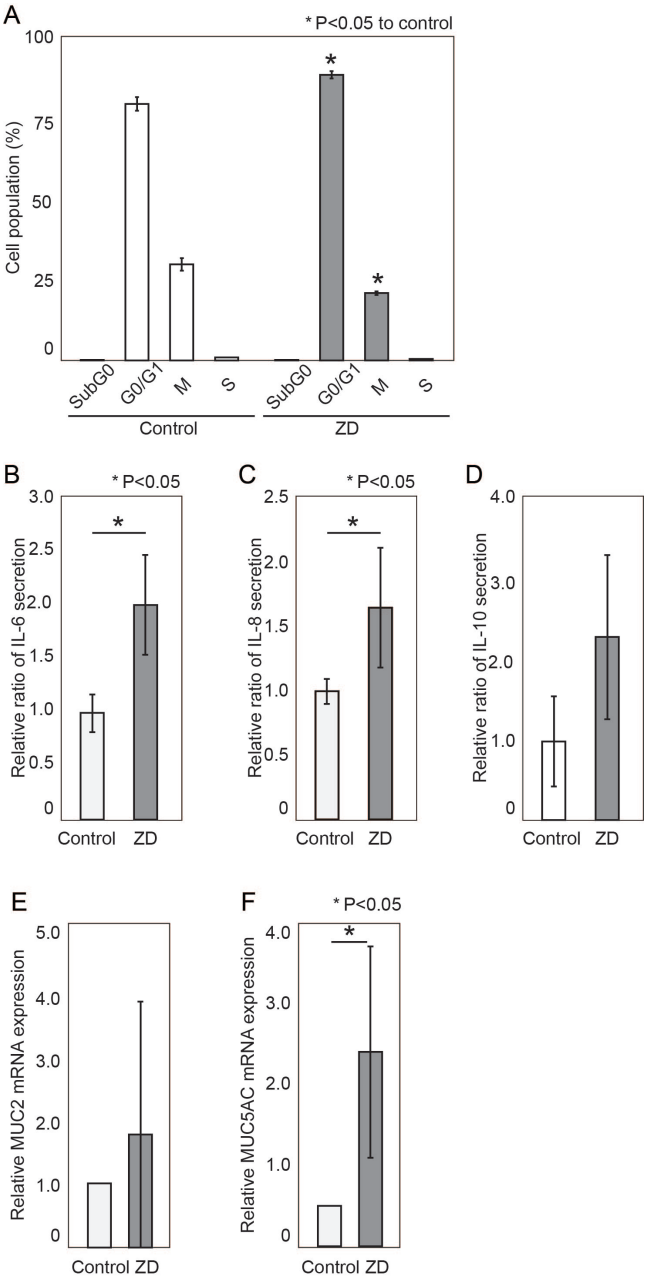

Figure 2. Zinc depletion of HNECs reduces cell proliferation, increases cell toxicity, and induces pro-inflammatory cytokines and Mucin 5AC. A) HNECs were incubated for 48 hours in zinc depleted (ZD) or control medium and percentage of cell populations were calculated. B-D) The ZD HNECs were incubated for 48 hours and the supernatants were subjected to ELISA for IL-6 (B), IL-8 (C), and IL-10 (D). Each protein level was standardized to the number of viable cells, and is indicated as a ratio to that of control cells, which were incubated in normal medium for the same period. E and F) The ZD HNECs were incubated for 48 hours followed by qPCR using primers for MUC2 (E) and MUC5AC (F). Data for A-F are means ± standard deviation (s.d.) of values from at least three independent experiments. *p<0.05, T-test with or without log-transformation.

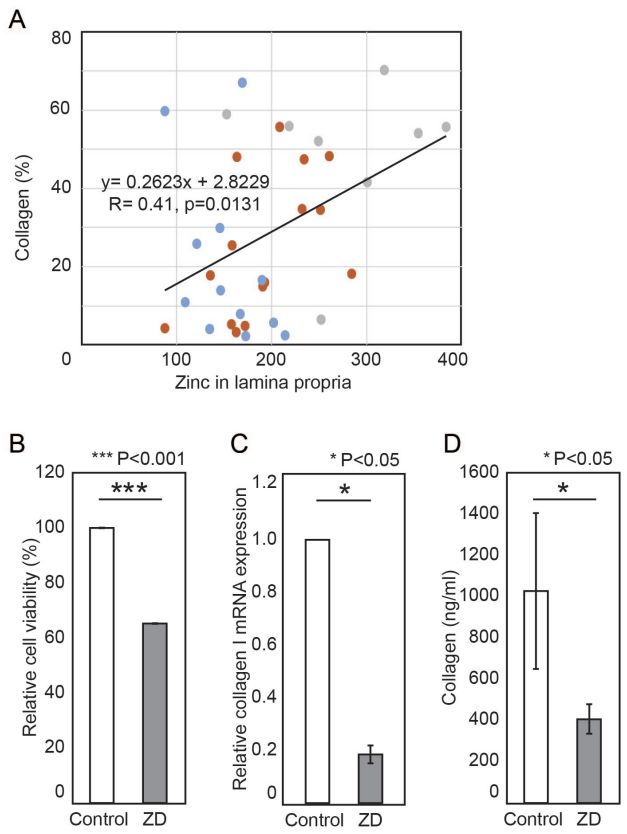

Figure 3. Low labile zinc levels positively correlate with tissue collagen in nasal mucosa. A) Pearson's correlation coefficient between collagen area and Zinquin fluorescence is shown in a scatter diagram. Collagen area in lamina propria were expressed as a percentage of the total lamina propria area. Skyblue, orange, and gray points stand for CRSwNP, CRSsNP and control, respectively. B-D) Fibroblasts from human nasal mucosa were incubated for 5 days in zinc depleted (ZD) medium followed by LDH assay (B) and qPCR analysis (C), and the supernatants were subjected to ELISA assay for collagen protein levels, normalized to the number of viable cells (D). Relative collagen I mRNA expression was determined by normalizing to untreated control cells and to GAPDH. Data are means ± standard deviation (s.d.) of values from three independent experiments. P values for indicated comparisons to control cells were determined by T test, *p<0.05 **p<0.01 ***p<0.001.

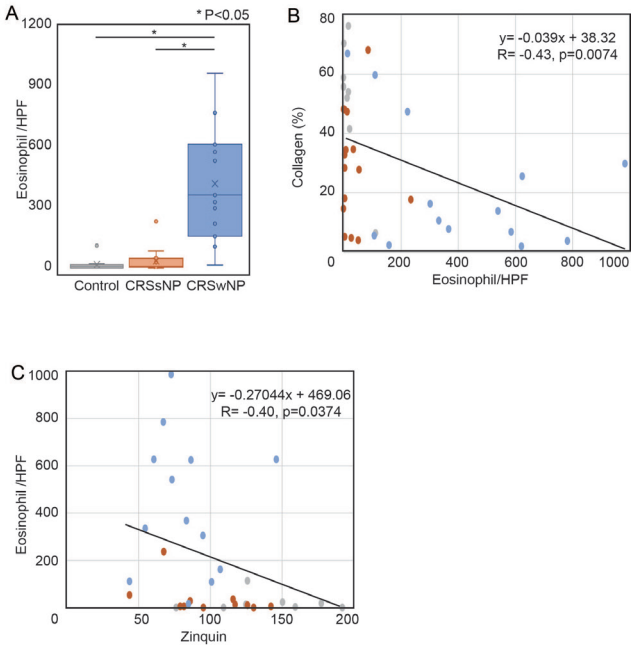

Figure 4. Eosinophil infiltration negatively correlates with collagen and zinc levels in nasal mucosa. A) Eosinophil counts in the lamina propria was compared between the different patient groups. *p<0.05, Kruskal-Wallis test. B) Eosinophil count and collagen area as percentage to the whole area and C) Zinquin fluorescence intensity and eosinophil count was shown in a scatter diagram with correlations tested using Pearson's correlation coefficient.

Zinc levels are increased in mucus in sites of inflammation in CRS patients

Zinc levels were measured in the serum and mucus collected from the inferior meatus or from the area of inflammation in controls (n=5), CRSsNP (n=10) and CRSwNP (n=5) patients. No significant differences in serum and inferior meatus zinc levels were observed amongst the groups (Supplementary Figure 2 and results not shown). In contrast, zinc levels were significantly increased in mucus samples collected from the area of inflammation in CRS patients compared to zinc levels in mucus samples collected from the inferior meatus (P=0.0243, Figure 1D).

Zinc depletion of HNECs reduces cell proliferation and cell viability, and induces pro-inflammatory cytokines and Mucin 5AC

Next, we investigated the effect of zinc depletion of primary human nasal epithelial cells (HNECs) on cell proliferation and cell viability. We established zinc depleted cells by culturing HNECs in zinc depleted medium for 48 hours and confirmed low intracellular labile zinc levels using zinquin fluorescence assays (Supplementary Figure 3A and 3B). Cell cycle analysis showed increased cell populations in the G0/G1 phase and decreased cell populations in the M-phase when comparing zinc depleted cells with controls, indicating zinc depletion reduced HNEC cell proliferation (p<0.05) (Figure 2A). In addition, the cell viability of zinc-depleted cells was significantly decreased (p<0.05) (Supplementary Figure 3C). Next, we investigated how zinc depletion affected cytokine expression/secretion by HNECs. ELISA assays showed a significant increase in IL-6 and IL-8 protein levels but not IL-10 in zinc depleted cells compared to control cells (Figure 2B, C, and D). qPCR showed MUC5AC mRNA expression was significantly induced in zinc depleted cells compared to controls (p=0.03), whilst MUC2 mRNA expression was not (Figure 2E and F).

Mucosal zinc levels are positively correlated with tissue collagen levels

In CRSwNP patients mucosal collagen levels were significantly reduced [7,8]. The homeostasis of collagen depends on a finely tuned balance of synthesis and degradation, and involves several zinc-dependent molecules [23-25]. We examined a possible relationship between tissue collagen levels and mucosal zinc levels. There was a significant positive correlation between collagen levels and labile zinc levels within the mucosa (R=0.41, P=0.013) (Figure 3A).

Reduced zinc levels decrease collagen production in primary human nasal fibroblasts

We then investigated how zinc depletion affected the synthesis and secretion of collagen in primary fibroblasts isolated from the nasal mucosa. Zinc depleted fibroblasts showed reduced cell viability compared to controls (Figure 3B). qPCR analysis and ELISA assays using zinc depleted fibroblasts showed significantly reduced mRNA expression levels and protein secretion of collagen I (Figure 3C and D).

Eosinophil infiltration correlates with tissue collagen and labile zinc levels

One of the most prominent characteristics of recalcitrant CRSwNP is eosinophil infiltration within the nasal mucosa [26]. We investigated possible correlations between collagen levels, eosinophil infiltration and intramucosal zinc levels. Eosinophil counts per HPF (0.035 mm^2) were significantly higher in CRSwNP than control and CRSsNP (Figure 4A, p=0.0046 to control and p=0.0146 to CRSsNP). There was a negative correlation between eosinophil counts and collagen levels (Figure 4B, R=-0.43, p=0.0074). In addition, significant negative correlations were found between eosinophil counts and zinquin levels (Figure 4C, R=-0.40, p=0.0374). We also found similar significant correlations among collagen levels, zinquin levels and all infiltrating inflammatory cells (correlations between inflammatory cells and collagen levels; R=-0.31, p=0.036, and inflammatory cells and zinquin levels; R=-0.49, p=0.0045, Supplementary Figure 4A and B).

Discussion

This study is the first to link reduced mucosal and increased mucus zinc levels in CRSwNP patients with a reduction in collagen deposition and increased eosinophil infiltration. Moreover, our study indicates zinc depletion reduces collagen synthesis and secretion by fibroblasts and increases pro-inflammatory cytokine production by HNECs in vitro.

Zinc is one of the most important trace elements in the human body and has a critical role in maintaining biochemical and physiological balance at the molecular, cellular and systemic level [10]. Zinc depletion affects multiple organs and causes defective immune function and impaired wound healing. A number of studies have indicated a detrimental role of zinc depletion in lower airway inflammation where it correlates with asthma severity [13,14] and induces a Th2- immune polarization with infiltration of eosinophils [15,27]. Importantly, zinc supplementation can have positive effects on symptom scores and lung function parameters in children with asthma [28], indicating the therapeutic potential of normalizing zinc homeostasis in the context of inflammatory conditions. Zinc depletion also causes hyperpermeability of the airway epithelium [12,29] and zinc homeostasis is critical for normal collagen synthesis [30-33]. Whilst characteristic for zinc depletion, a Th2-dominant immune response with infiltration of eosinophils, hyperpermeability of the mucosa and reduced collagen content are also hallmarks of CRSwNP [34,35]. In this study, mucosal zinc depletion in the context of CRSwNP significantly correlated with levels of inflammation, tissue eosinophilia and reduced collagen content. TGF-β signaling pathway is critical for the synthesis and deposition of collagen and several molecules within this pathway are dependent on zinc [23-25]. In CRSwNP, the TGF-β signal is thought to be attenuated with low expression levels of TGF-β1, TGF-β receptor II and SMAD proteins [9,36]. Taken together with our results, it can be hypothesized that low mucosal zinc levels might contribute to attenuation of TGF-β signaling and reduced collagen production in CRSwNP patients. Although the consequence of mucosal zinc depletion and low collagen content in the pathophysiology of CRSwNP is unknown, collagen and the factors promoting its synthesis including zinc and TGF-β signaling could be new therapeutic targets for the treatment of CRSwNP.

Given the normal serum zinc levels found in this and previous studies [12], the pathogenesis of mucosal zinc depletion is unclear. Allergy and infection, frequently associated with CRS[37], might contribute to mucosal zinc depletion. Stimulation with lipopolysaccharide, a bacterial product derived from the cell wall of gram-negative microbes, has been reported to decrease intracellular labile zinc levels in dendritic cells [38]. Also allergic inflammation leads to a decrease in intracellular zinc levels in airway epithelium of ovalbumin-treated mice [39]. In this study, increased mucus zinc levels were found at the actual site of inflammation but not in mucus harvested from the inferior meatus of the same patients. This finding could imply a localized increased zinc release via nasal discharge in sites of inflammation that could potentially contribute to the decreased mucosal zinc levels observed. Hypersecretion of mucus from the nasal epithelium is a hallmark of CRSwNP and mucin, one of the principal components of mucus, has been reported to efficiently bind zinc [40,41]. Intense intracellular labile zinc staining was indeed observed within the submucosal glands, the site of mucus production in the nasal mucosa. Taken together with significantly higher MUC5AC mRNA expression and inflammatory cytokine release in zinc depleted cells, we hypothesize the presence of

a vicious cycle where increased mucus and zinc-binding mucin production in the context of inflammation results in a net loss of zinc from the mucosa. The resulting mucosal zinc depletion contributes to increased inflammation, Th2-polarisation and mucin expression and release, further promoting relative zinc loss (Supplementary Figure 5).

Our results indicate normal serum zinc levels, hence the absence of zinc deficiency, in CRS patients. It is therefore unlikely that zinc supplementation would help alleviate the inflammatory process in those patients. Furthermore, whereas a causative role of mucosal zinc depletion in the pathogenesis of CRS could not be demonstrated, our results indicate an association of local mucosal zinc depletion in Th2 immune polarization and reduction in collagen content in the context of CRS. Whilst further research is required, this study implies that mucosal zinc depletion marks CRSwNP and that promoting mucosal zinc homeostasis could help reduce inflammation in CRS patients.

Conclusion

Mucosal zinc depletion associates with tissue eosinophilia and collagen depletion in CRSwNP and induces pro-inflammatory cytokine expression and reduction of collagen synthesis in vitro. New therapeutic strategies promoting mucosal zinc homeostasis could therefore help alleviate inflammation in CRS.

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Authorship contribution

MS, YN, and SV designed the experiments. AJP, AH, and PJW supervised the project. MS and MR performed most of the experiments and compiled the data. TJL and BJ involved in experiments of qPCR. CC and SK performed sample preparation. MS, MR, TS, and SV wrote the manuscript. All authors provided feedback on the manuscript.

Conflict of interest

None of the authors have any conflicts of interest or financial disclosures that are relevant to this study.

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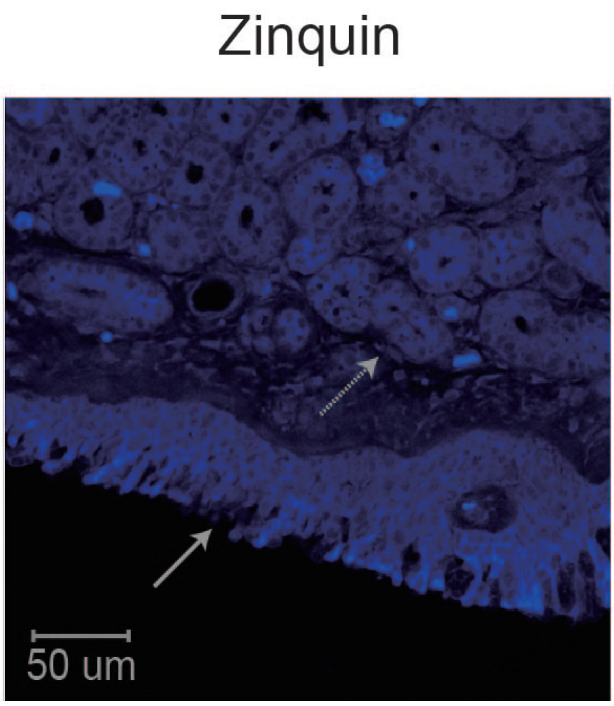
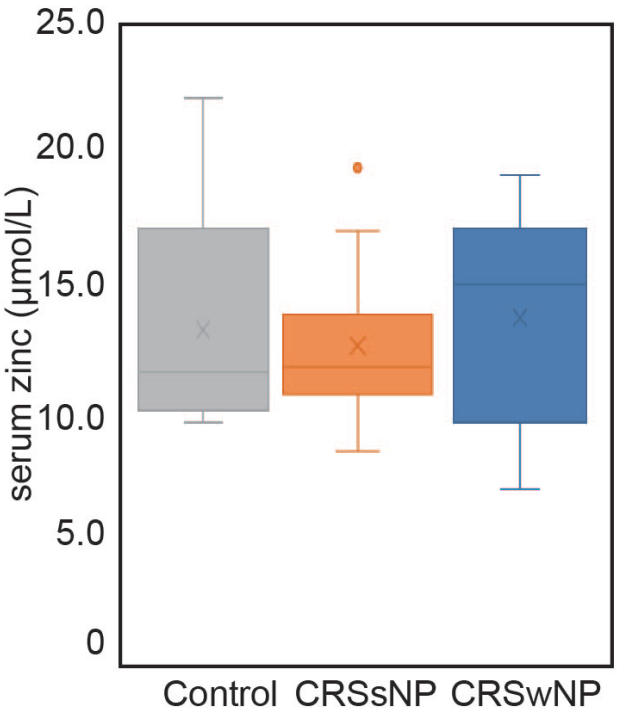
This paper contains supplementary materials online: at www.rhinologyjournal.org

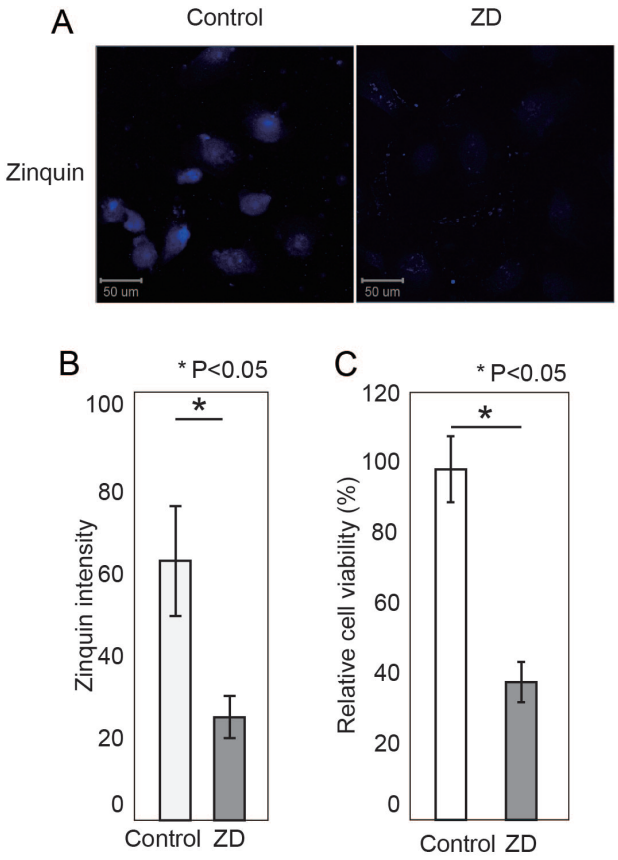
SUPPLEMENTARY DATA



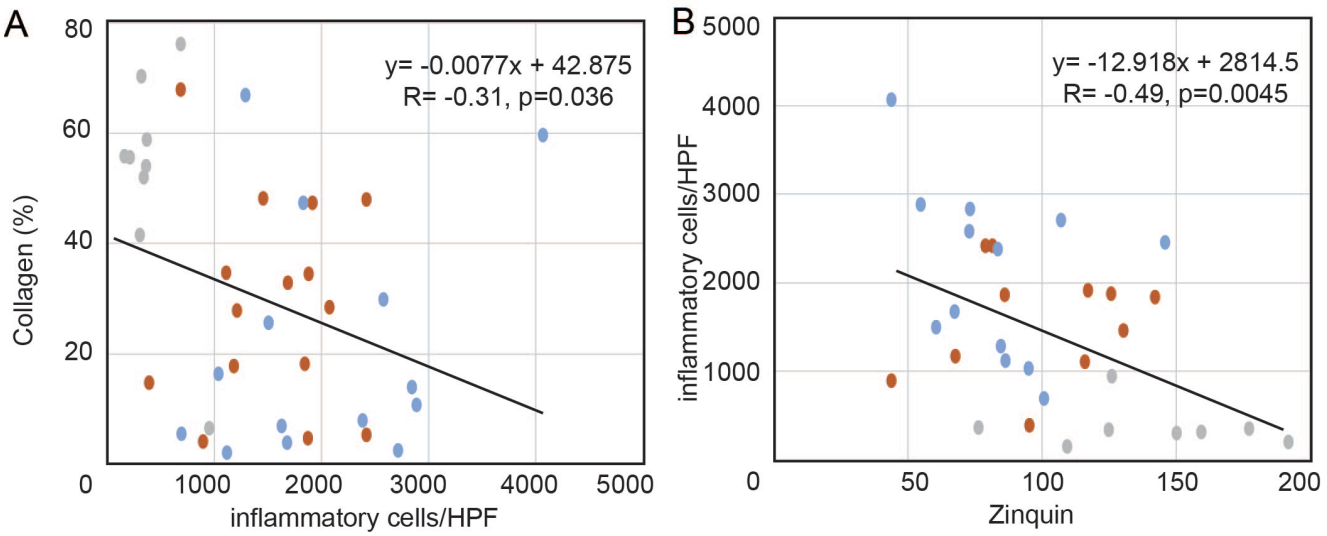
Supplementary figure 1.
Immunofluorescence of representative nasal mucosa including surface epithelium (gray arrow) and nasal glands and ducts (dotted arrow). Zinquin stains labile zinc blue.



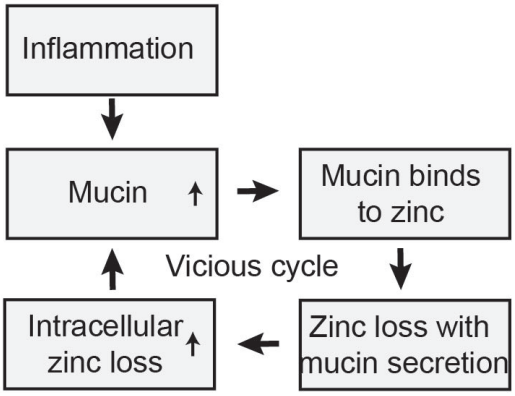
Supplementary figure 2. Serum zinc levels were compared between the different patient groups. Kruskal-Wallis test, p>0.05.



Supplementary figure 3. A and B. HNECs were incubated for 48 hours in zinc depleted (ZD) or control media followed by immunofluorescence using zinquin. C. Cell viability of the ZD HNECs relative to control cells, determined by LDH assay. Data are means ± standard deviation (s.d.) of values from at least three independent experiments. P values for indicated comparisons were determined by t-test, *p<0.05.



Supplementary figure 4. A. Inflammatory cell count/High Power Field and collagen area as a percentage to the whole area were shown in a scatter diagram. B. Zinquin fluorescence intensity and inflammatory cell count/High Power Field were shown in a scatter diagram. The correlations were tested using Pearson's correlation coefficient.



Supplementary figure 5. Possible vicious cycle of mucin production and intracellular zinc levels.