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Immunological variations in Waldeyer's ring and oral mucosa during oromucosal treatment with lactoferrin, colostrum, zinc and vitamin D

Simonetta Masieri[1], Francesco Frati[2,*], Carlo Cavaliere[3], Sofia Guida[4], Giulio Torello[5], Giuseppe Di Cara[6], Giovanni D'Erme[3], Caruso Armone[7] and Fabrizio Mainiero[8].

[1] Department of Odontostomatologic and Maxillofacial Sciences, Sapienza University, Rome, Italy.
[2] Department of Medicine and Surgery, Otorhinolaryngology Section, University of Perugia, Perugia, Italy.
[3] Department of Sense Organs, Sapienza University, Rome, Italy.
[4] UOC of Immunopathology, Polyclinic Umberto I, Sapienza University, Rome, Italy.
[5] TL Pharma Consulting, Pescara, Italy
[6] Department of Pediatrics, University of Perugia, Perugia, Italy.
[7] AIAS Afragola, Naples, Italy.
[8] Department of Experimental Medicine, Sapienza University, Rome, Italy.

Abstract: Micronutrients and other dietary components have shown in several studies variable ranges of immunological activity, resulting in an anti-infective response which may play a role in the burden of upper and lower airways infections in vulnerable people, childhood and older age. [1] The aim of our study was to evaluate the modifications in IFN γ and TGF β production, after oral supplementation with a mixture of lactoferrin, colostrum, zinc and vitamin D in patients with chronic rhinopharyngitis. After 30 days of treatment the active treatment group showed a significant ($p<0.01$) variation in the lingual tonsil of IFN γ (12.8 ± 6.2 vs 17.3 ± 9.9 pg/mL) and TGF β (71.6 ± 24.4 vs 101.6 ± 30.8 pg/mL). Similar results were evident in oral mucosa both for IFN γ (9.3 ± 5.9 vs 12.3 ± 6.4 pg/mL) and TGF β (65.7 ± 15.5 vs 82.2 ± 12.5 pg/mL). The evidence of immunological modifications after treatment with a nutraceutical product, with the possibility to detect anti-infective cytokines directly in Waldeyer's ring may play an important role in supporting the evidence of clinical efficacy studies.

Keywords: lactoferrin; immunoceuticals; nutraceuticals; recurrent respiratory infections

1. Introduction

Different environmental factors such as pollution, infections, and diet may play a pivotal role in the development of the immune system. In recent years several studies have evidenced that food components may act as possible modifiers of the immune response, through ingestion and direct contact with specific active sites as the Waldeyer's tonsillar ring, or nasal-associated lymphoid tissue (NALT) [2]. The lymphoid tissue of Waldeyer's ring is the gateway of the respiratory and alimentary tract and is involved also in the response of the mucosa-associated lymphoid tissue (MALT) towards several potential pathogens and allergens [3,4].

Pharyngeal, palatine and lingual tonsils possibly play different but synergic roles according to their site and lymphatic drainage. These specialized lymphoepithelial regions regulate the locoregional immune response to a variety of antigens from foods, environment and pathogens, contributing to a wide array of processes involving innate and adaptive response. While pharyngeal tonsil, with its location

in the nasopharynx and its ciliated pseudostratified epithelium, has a specific role in contacting respiratory agents and is involved in the clearance of upper airways pathogens and in the development of chronic respiratory conditions, palatine and lingual tonsils, covered with a stratified non-keratinized squamous epithelium, are able to interact also with several dietary antigens and food components, which may impact with different roles in the immune system regulation and differentiation, both influencing innate and acquired response[5,6].

A peculiar aspect of the Waldeyer's ring is the presence of particular anatomical structures: the crypts, fundamental areas for the interaction of the immune system both with respiratory pathogens and micro-nutrients taken during the normal diet; in this case the Waldeyer's component involved is the lingual tonsil, the obligatory transit area for all foods during the ingestion.

The crypts are multiple antigen-retaining areas, that greatly increase the surface of contact between food components and the underlying cells. The reticulated epithelium lining the tonsillar crypts is probably an antigen entry portal, specialized in transporting antigens to the subepithelial tissue after food consumption.

Nutrients, and in particular aminoacids, are used as substrate for the production of immunoglobulins, enzymes and cytokines, thus relating a good nutritional status with a physiological expression and maintenance of the immune response to bacterial and viral infections. Micronutrients overlap every stage of this immune response supporting mucosal barrier integrity, growth and differentiation of NK-cells, B lymphocytes and T-reg cells, and enhancing chemotactic and antibacterial activity of neutrophils and macrophages [6,7]. However, while there is a wide support on the specific role of a single nutritional component in each stage of this response, the activity of specific mixtures of different dietary components has not yet been evaluated [7].

The aim of our study was to evaluate the in situ immunological antimicrobial response (lingual tonsil and oral mucosa) after oral supplementation with a mixture of well-known food components whose effects have been specifically defined in several studies: lactoferrin, colostrum, zinc and vitamin D [8,9]. Zinc is involved both in the regulation of cellular response and protection from oxidative and inflammatory stress [10]. Vitamin D promotes antigen processing by dendritic cells and synthesis of antimicrobial molecules, such as defensins [11]. Colostrum shows a high antioxidative activity and promotes phagocytic activity while the antimicrobial activity of lactoferrin has been well evidenced with its iron sequestering property being particularly effective against bacteria [12–18].

The possible immunological role of such mixture was investigated detecting modifications in the local production of IFN γ and TGF β, cytokines involved in the innate and adaptive response against viral, bacterial and protozoan infections.

2. Materials and Methods

2.1. Characteristics of patients

In order to reflect a real-life approach, 13 consecutive patients with chronic rhinopharyngitis (aged between 18 and 50, 10 women and 3 men) evaluated as outpatients in the ENT clinic of Università La Sapienza of Rome were included in our study. Diagnosis of chronic rhinopharyngitis was defined evaluating personal history, and clinical evaluation during acute symptoms according together with sinonasal imaging. Patients with positive skin prick tests for aeroallergens, with current systemic therapy with corticosteroids, and severe systemic diseases were

excluded from the study[19,20].10 patients were enrolled to self-administer a nutraceutical mixture of lactoferrin 100 mg, zinc 10 mg, colostrum 200 mg and vitamin D_3 10 mcg ("Waldimmun" oral sticks BIODEMIA), once a day for 30 days, at sublingual level. Three patients, who refused the nutraceutical mixture for economic reasons, were used as control group and were suggested to self-administer oral paracetamol, when needed. Treatment with oral medications was discontinued at least 10 days prior to the sampling procedure.

2.2. Oral and lingual tonsil brushing sampling and detection

Oral and lingual tonsil mucosa samples were collected at baseline (T0) and after 30 days of treatment (T1). For each patient, two separate specimens were obtained, one from the brushing of the lingual tonsil and one from the brushing of oral mucosa. The latter sample was collected with the aid of a nasal endoscope. Samples were obtained through a disposable curette with a small distal cup (Rhino-probeTM, Arlington Scientific, Inc., Springville, UT, USA) and stored at -80°C in PBS until determination. IFN γ and TGF β levels were detected with ELISA method (Human IFN-gamma ELISA and Human TGF-beta ELISA, Eurosiotal, Trieste, Italy).

2.3. Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (SPSS, Chicago, IL, USA). The Kolmogorov–Smirnov test was used to evaluate the normality of distribution of each continuous variable and Mann–Whitney test was then used to compare each variable between T0 and T1; p values of < 0.05 were considered statistically significant.

3. Results

Patients in the control group did not show any significant variation in the 30 days follow-up period in lingual tonsil for IFN γ (20.4 ± 5.8 vs 19.7 ± 4.9 pg/mL) and TGF β (63.1 ± 20.2 vs 61.8 ± 20.1 pg/mL) and in the oral mucosa (IFN γ16.8 ± 9.7 vs 16.6 ± 9.3 pg/mL; TGF β 52.2 ± 18.7 vs 52.8 ± 18.9 pg/m pg/mL). (Table 1 and 2)

Patient in the active treatment group showed a significant ($p<0.01$) variation in lingual tonsil of IFN γ (from 12.8 ± 6.2 at baseline to 17.3 ± 9.9 pg/mL) and TGF β (from 71.6 ± 24.4 at baseline to 101.6 ± 30.8 pg/mL) after the 30-days treatment. Similar results were evident in oral mucosa both for IFN γ (from 9.3 ± 5.9 at baseline to 12.3 ± 6.4 pg/mL) and TGF β (from 65.7 ± 15.5 at baseline to 82.2 ± 12.5 pg/mL) after the 30-days treatment.

(Table 1 and 2)

Table 1. IFN γ and TGF β levels in lingual tonsil specimens.

	Lingual tonsil		
IFN γ (pg/mL)	**T0**	**T1**	sig. (p)
Group 1 (treated)	12.8 ± 6.2	17.3 ± 9.9	<0.01
Group 2 (control)	20.4 ± 5.8	19.7 ± 4.9	NS (0.28)
TGF β (pg/mL)	T0	T1	sig. (p)
Group 1 (treated)	71.6 ± 24.4	101.6 ± 30.8	<0.01
Group 2 (control)	63.1 ± 20.2	61.8 ± 20.1	NS (0.65)

Table 2. IFN γ and TGF β levels in oral mucosa specimens.

	Oral mucosa		
IFN γ (pg/mL)	**T0**	**T1**	sig. (p)
Group 1 (treated)	9.3 ± 5.9	12.3 ± 6.4	<0.01
Group 2 (control)	16.8 ± 9.7	16.6 ± 9.3	NS (0.11)
TGF β (pg/mL)	T0	T1	sig. (p)
Group 1 (treated)	65.7 ±15.5	82.2 ±12.5	<0.01
Group 2 (control)	52.2 ±18.7	52.0 ±18.9	NS (0.18)

None of the patients in group 1 reported any significant side effects or adverse events during the follow-up period.

4. Discussion

Relevant evidences in the last years have shown the clinical efficacy of dietary supplements containing separately lactoferrin, colostrum, zinc and vitamin D. However, the real mechanisms of action involved in the modulation of immune response are still on debate [21].

Because of its location, Waldeyer's ring acts as a primary defense line against respiratory and swallowed pathogens, which are sampled in this region, and is also exposed to nutritional components which may interfere or modulate the immune response against infectious and other inflammatory diseases, such as allergies. The tonsils, lymph follicles and lymph nodes of Waldeyer's ring are anatomically structured to increase the surface area of contact between food components and the innate and adaptive immune cells.

Several findings from animal studies suggest that the epithelium of lingual tonsil seems to be specialized in transporting antigens to the subepithelial tissue within few minutes after food consumption, where the processing of antigens will stimulate naïve T and B lymphocytes [4,22].

Modern nutraceutical approach, according to which a food or part of a food may provide medical benefits, including the prevention and/or treatment of a disease or illness, suggests that several food components or dietary supplements could potentiate innate and adaptive immunity against several environmental stimuli, especially against infectious pathogens, through the activation of mucosal and submucosal cells located in several sites of Waldeyer's ring [2].

Such hypothesis, however, although theoretically fascinating, still lacks immunological evidences. Thus, our preliminary study was aimed at detecting a possible immunological effect of this approach, sampling IFN γ and TGF β, cytokines involved in the defence mechanisms against infectious diseases, and their variation after a nutraceutical treatment.

With regard to immunological modifications directly in the oral mucosa, our data confirm once again, the involvement of this anatomical area in the complex mechanisms of immunological interaction during the food ingestion.

Oral mucosa itself has not only protective functions as a primary barrier for the contact of antigens from foods or pathogens with the gastrointestinal system, but also interacts constantly with the local immune system contributing to the effective response against virus and bacteria. Peptides and molecules from dietary components, oral microbiota and pathogens, which may present outside the oral epithelium may contact antigen presenting cells in the subepithelial region through a paracellular pathway or via a transcellular route. This interaction is fundamental in the maintenance of the homeostasis of oral mucosa against the

constant exposure to the various environmental signals, and particularly in optimizing the innate and adaptive defense mechanisms.

Upper and lower airways infections are one of the most important health topics, especially during autumn and winter, and several epidemiological data suggest a nearly worldwide burden of cases that affect vulnerable people, childhood and older age, with an increase of antibiotic misuse and hospitalization [13].

Vaccination strategies have been able to better control incidence and mortality trends against specific diseases but a wide-angle strategy is still lacking [23].

Several studies evaluating and reviewing the activity of immunostimulants, which have been gradually introduced for the prevention and treatment of recurrent respiratory tract infections, still fail to quantify their real pharmacological and immunological effect [24,25].

A nutraceutical approach to this problem may open a new path of treatment with high safety profile molecules and potentially relevant clinical results, both in prevention of recurrent respiratory infections and as adjuvant during an acute respiratory illness.

However, among the different products commercialized for such purpose, the main question is the definition of which products may really be promising from an immunological point of view.

The possibility to detect anti-infective cytokines directly in Waldeyer's ring may play an important role, in order to define possible efficacy biomarkers which may support the evidence of clinical efficacy studies.

In our innovative work, the evidence of immunological modifications both in oral mucosa and in lingual tonsil after a 30-days treatment with a nutraceutical product containing lactoferrin, colostrum, zinc and vitamin D may be the immunological premise to studies evaluating safety profiles, clinical efficacy, and treatment schedules.

To conclude, a peculiar aspect of our work is relating to the evidence that important immunological modifications during feeding occur early and directly at the level of oral mucosa and the Waldeyer's ring in conjunction with the ingestion of various foods or fundamental nutraceutical components.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Marshall, J.S.; Warrington, R.; Watson, W.; Kim, H.L. An Introduction to Immunology and Immunopathology. *Allergy Asthma Clin. Immunol. Off. J. Can. Soc. Allergy Clin. Immunol.***2018**, *14*, 49, doi:10.1186/s13223-018-0278-1.
2. Govers, C.; Calder, P.C.; Savelkoul, H.F.J.; Albers, R.; van Neerven, R.J.J. Ingestion, Immunity, and Infection: Nutrition and Viral Respiratory Tract Infections. *Front. Immunol.***2022**, *13*, 841532, doi:10.3389/fimmu.2022.841532.
3. Masieri, S.; Trabattoni, D.; Incorvaia, C.; De Luca, M.C.; Dell'Albani, I.; Leo, G.; Frati, F. A Role for Waldeyer's Ring in Immunological Response to Allergens. *Curr. Med. Res. Opin.***2014**, *30*, 203–205, doi:10.1185/03007995.2013.855185.

4. Masieri, S.; Incorvaia, C.; Cavaliere, C.; Di Cara, G.; Frati, F. Is Lingual Tonsil a Preferential Target for Processing Sublingually Administered Materials? *J. Biol. Regul. Homeost. Agents***2017**, 3.
5. van Daal, M.T.; Folkerts, G.; Garssen, J.; Braber, S. Pharmacological Modulation of Immune Responses by Nutritional Components. *Pharmacol. Rev.***2021**, *73*, 198–232, doi:10.1124/pharmrev.120.000063.
6. Gombart, A.F.; Pierre, A.; Maggini, S. A Review of Micronutrients and the Immune System-Working in Harmony to Reduce the Risk of Infection. *Nutrients***2020**, *12*, 236, doi:10.3390/nu12010236.
7. Pecora, F.; Persico, F.; Argentiero, A.; Neglia, C.; Esposito, S. The Role of Micronutrients in Support of the Immune Response against Viral Infections. *Nutrients***2020**, *12*, 3198, doi:10.3390/nu12103198.
8. Ma, T.; D, S. TGF-β Activation and Function in Immunity. *Annu. Rev. Immunol.***2014**, *32*, doi:10.1146/annurev-immunol-032713-120257.
9. Schroder, K.; Hertzog, P.J.; Ravasi, T.; Hume, D.A. Interferon-Gamma: An Overview of Signals, Mechanisms and Functions. *J. Leukoc. Biol.***2004**, *75*, 163–189, doi:10.1189/jlb.0603252.
10. Prasad, A.S.; Bao, B.; Beck, F.W.J.; Kucuk, O.; Sarkar, F.H. Antioxidant Effect of Zinc in Humans. *Free Radic. Biol. Med.***2004**, *37*, 1182–1190, doi:10.1016/j.freeradbiomed.2004.07.007.
11. Teymoori-Rad, M.; Shokri, F.; Salimi, V.; Marashi, S.M. The Interplay between Vitamin D and Viral Infections. *Rev. Med. Virol.***2019**, *29*, e2032, doi:10.1002/rmv.2032.
12. Berthon, B.S.; Williams, L.M.; Williams, E.J.; Wood, L.G. Effect of Lactoferrin Supplementation on Inflammation, Immune Function, and Prevention of Respiratory Tract Infections in Humans: A Systematic Review and Meta-Analysis. *Adv. Nutr. Bethesda Md***2022**, *13*, 1799–1819, doi:10.1093/advances/nmac047.
13. Bernstein, J.M. Waldeyer's Ring and Otitis Media: The Nasopharyngeal Tonsil and Otitis Media. *Int. J. Pediatr. Otorhinolaryngol.***1999**, *49 Suppl 1*, S127-132, doi:10.1016/s0165-5876(99)00146-9.
14. Hunter, J.; Arentz, S.; Goldenberg, J.; Yang, G.; Beardsley, J.; Myers, S.P.; Mertz, D.; Leeder, S. Zinc for the Prevention or Treatment of Acute Viral Respiratory Tract Infections in Adults: A Rapid Systematic Review and Meta-Analysis of Randomised Controlled Trials. *BMJ Open***2021**, *11*, e047474, doi:10.1136/bmjopen-2020-047474.
15. Saad, K.; Abo-Elela, M.G.M.; El-Baseer, K.A.A.; Ahmed, A.E.; Ahmad, F.-A.; Tawfeek, M.S.K.; El-Houfey, A.A.; Aboul Khair, M.D.; Abdel-Salam, A.M.; Abo-Elgheit, A.; et al. Effects of Bovine Colostrum on Recurrent Respiratory Tract Infections and Diarrhea in Children. *Medicine (Baltimore)***2016**, *95*, e4560, doi:10.1097/MD.0000000000004560.
16. Sassi, F.; Tamone, C.; D'Amelio, P. Vitamin D: Nutrient, Hormone, and Immunomodulator. *Nutrients***2018**, *10*, 1656, doi:10.3390/nu10111656.
17. Shin, K.; Oda, H.; Wakabayashi, H.; Yamauchi, K.; Abe, F. Effects of Lactoferrin on the Production of Interferon-λ by the Human Intestinal Epithelial Cell Line HT-29. *Biochem. Cell Biol. Biochim. Biol. Cell.***2017**, *95*, 53–56, doi:10.1139/bcb-2016-0031.
18. Wang, T.-T.; Nestel, F.P.; Bourdeau, V.; Nagai, Y.; Wang, Q.; Liao, J.; Tavera-Mendoza, L.; Lin, R.; Hanrahan, J.W.; Mader, S.; et al. Cutting Edge: 1,25-Dihydroxyvitamin D3 Is a Direct Inducer of Antimicrobial Peptide Gene Expression. *J. Immunol. Baltim. Md 1950***2004**, *173*, 2909–2912, doi:10.4049/jimmunol.173.5.2909.
19. Fokkens, W.J.; Lund, V.J.; Hopkins, C.; Hellings, P.W.; Kern, R.; Reitsma, S.; Toppila-Salmi, S.; Bernal-Sprekelsen, M.; Mullol, J.; Alobid, I.; et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2020. *Rhinology***2020**, *58*, 1–464, doi:10.4193/Rhin20.600.
20. Stephenson, K.N. Acute and Chronic Pharyngitis across the Lifespan. *Lippincotts Prim. Care Pract.***2000**, *4*, 471–489.
21. Tieu, S.; Charchoglyan, A.; Wagter-Lesperance, L.; Karimi, K.; Bridle, B.W.; Karrow, N.A.; Mallard, B.A. Immunoceuticals: Harnessing Their Immunomodulatory Potential to Promote Health and Wellness. *Nutrients***2022**, *14*, 4075, doi:10.3390/nu14194075.
22. Frati, F.; Masieri, S.; Incorvaia, C.; Buttafava, S.; Mascarell, L.; Moingeon, P. Enhancing Sublingual Immunotherapy by Systemic or Local Factors: A Role for Vitamin D and Lingual Tonsil? *Allergy Asthma Proc.***2017**, *38*, 3, doi:10.2500/aap.2017.38.4020.
23. Jefferson, T.; Rivetti, A.; Di Pietrantonj, C.; Demicheli, V. Vaccines for Preventing Influenza in Healthy Children. *Cochrane Database Syst. Rev.***2018**, *2*, CD004879, doi:10.1002/14651858.CD004879.pub5.
24. Chiappini, E.; Santamaria, F.; Marseglia, G.L.; Marchisio, P.; Galli, L.; Cutrera, R.; de Martino, M.; Antonini, S.; Becherucci, P.; Biasci, P.; et al. Prevention of Recurrent Respiratory Infections : Inter-Society Consensus. *Ital. J. Pediatr.***2021**, *47*, 211, doi:10.1186/s13052-021-01150-0.
25. Berber, A.; Del-Río-Navarro, B.E.; Reyes-Noriega, N.; Sienra-Monge, J.J.L. Immunostimulants for Preventing Respiratory Tract Infection in Children: A Systematic Review and Meta-Analysis. *World Allergy Organ. J.***2022**, *15*, 100684, doi:10.1016/j.waojou.2022.100684.