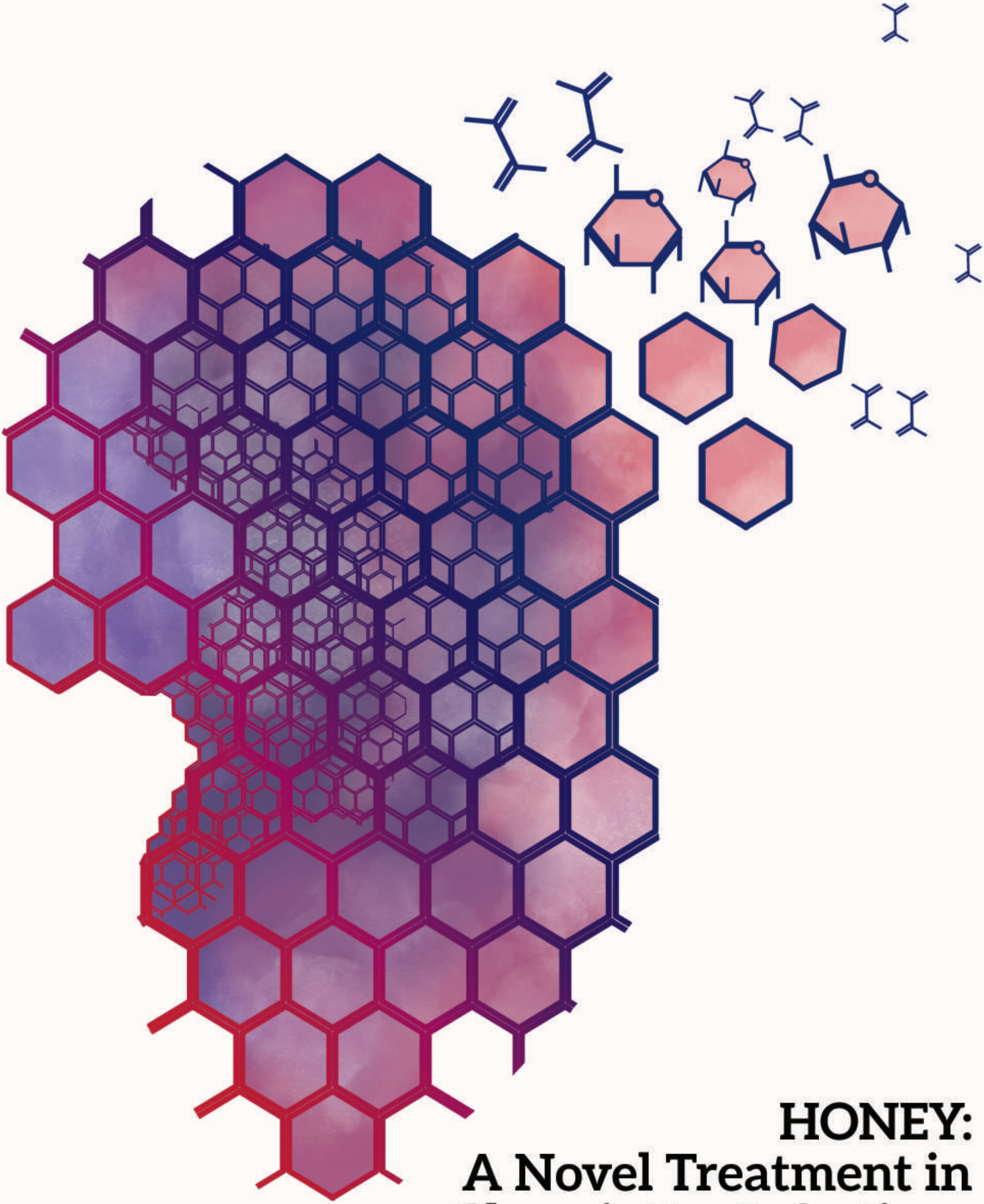


HONEY:
A Novel Treatment in Chronic Ear Infections

Darius Henatsch

Honey: A Novel Treatment In Chronic Ear Infections

Darius Henatsch

Honey: a novel treatment in chronic ear infections
C.D. Henatsch
Maastricht University

ISBN: 978-94-028-1626-6

Cover layout: Evelien Jagtman

Layout and print: Wendy Bour-van Telgen, Ipskamp Printing, Enschede

Financial support was kindly provided by Dos Medical BV / KNO-winkel.nl

Honey: A Novel Treatment In Chronic Ear Infections

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. R.M. Letschert
volgens het besluit van het College van Decanen,

in het openbaar te verdedigen

op vrijdag 6 september 2019 om 14.00 uur

door

Caspar Darius Henatsch
Geboren op 1 april 1984 te Keulen

Promotor
Prof. dr. R.J. Stokroos

Copromotor
Dr. J.J. Briedé

Beoordelingscommissie
Prof. dr. B. Kremer (voorzitter)
Prof. dr. S. Heeneman
Prof. dr. P.M. Steijlen
Prof. dr. J.H. Coert, Universitair Medisch Centrum Utrecht
Dr. R. Pennings, Radboud Universitair Medisch Centrum

CONTENTS

CHAPTER 1

-

General introduction

Chronic ear infections: a non-healing wound

Two conditions of the skin of the external ear canal and its vicinity, which often manifest as a chronic illness, are: eczematous external otitis and the discharging open mastoid cavity.

Eczematous external otitis is an inflammation of the external auditory canal, with symptoms of pruritus, erythema and scaling. Exacerbations are often accompanied by discharge and acute secondary bacterial infections[1]. General atopic dermatitis is very common and affects up to 3% of the adult population[2]. The pathophysiology of atopic eczema is an immunological sensitization, due to a malfunction of the skin, which enables allergens to penetrate the epidermal barrier[3]. An eczematous skin lesion is accompanied by *Staphylococcus aureus*[5] increased oxidative stress, decreased local antioxidants[4] and a colonization with Staphylococcus aureus[5]. This colonization and a reduced skin microbial diversity again influences allergic sensitization[6]. Patients with eczematous external otitis have more often asymptomatic food hypersensitivity[7], and about 30% have positive epicutaneous patch test results, leading to a higher incidence of recurrence[8].

Another chronic condition, located in the vicinity of the external ear canal is the chronically discharging open mastoid cavity. In patients with serious chronic suppurative otitis media and cholesteatoma surgical intervention is inevitable[9]. An option is the creation of an open mastoid cavity, by partial surgical removal of the petrous temporal bone, with exteriorization into the external auditory canal[10,11]. With this intervention, one big cavity is created to remove diseased tissue, to facilitate inspection and cleaning and to generate outflow of discharge[12]. After the surgical intervention, which can effect any age group[13], up to 20-60% of patients continue to have intermittent or persistent troublesome otorrhea[14,15], even with regular aural toilet and topical medication[13,16]. Different factors contribute to an unstable cavity: 1. Anatomical features of the cavity itself, as a high facial ridge or a small external meatus. 2. Persistent origin of the disease, as perforation of the eardrum or residual cholesteatoma. And importantly, patient factors that lead to (partial) failure of (re-)epithelialization[13]. Latter, insufficient epithelial healing and coverage leads to exudation, which in combination with keratin debris, is a good culture medium for bacterial growth[17]. Infection, mainly by *Pseudomonal* species[18,19], oxidative stress[20,21], and the formation of granulation tissue again hinder the formation of new epithelium[22].

Important factors in both types of chronic ear infections are interaction of cells of the immune system and bacterial infection. In this way these chronic ear conditions bear all characteristics of a chronic (non-healing) wound. Chronic wounds do not follow the physiological step-wise process of healing (inflammation, proliferation and maturation/remodeling)[23] and rather remain in an uncoordinated, self-sustaining state of inflammation[24]. For the transition from inflammation to repair phase, the removal from noxious stimuli, followed by the replacement of cytokines by growth factors, is an inescapable step in healthy wound healing[25]. The pathologic micro-milieu in the chronic, non-healing wound is composed of amongst others, bacterial colonization, increased oxygen radicals, pro-inflammatory cytokines and proteases[26].

The chronic wound: need for new antiseptic strategies

The discovery of penicillin by Sir Alexander Fleming in 1928 introduced a new era in the treatment of infections[27]. Unfortunately, bacterial resistance has increased during the last decades, which was partly caused by over-prescription. Nowadays, one can find pathogens that are resistant against nearly all antibiotics. This, in combination with the stagnation of the discovery of new antibiotic agents, is a new global challenge[28,29] and accentuates the need for more and novel antiseptic strategies.

The action of antiseptics differs to those of antibiotics. They often damage the surface of microbes and act bactericidal (killing of bacteria) or bacteriostatic (inhibition of growth of bacteria), with a broad spectrum of action[30]. Resistances to antiseptics are known as well, but to a much lesser extend[31]. The action of antibiotics is different. They mainly have single targets and a small spectrum of action[32]. Antiseptics can be divided in different classes as iodine-based, silver-based, honey-based, and others[30]. They can prevent bacterial resistance formation, lead to less allergic reactions[33] and can optimize individual therapy and prevent antibiotic overuse[34]. Based on the lack of powerful clinical studies, which combine different treatments, it is still not possible to advise a single superior antiseptic agent[35].

In the chronic ear conditions eczematous external otitis and the discharging open mastoid cavity, corticosteroids in combination with antibiotics are often used as a topical treatment. A problem mainly with chronic and recurrent use in both conditions are the formation of bacterial resistance[36,37], sensitization to compounds[38-41] and skin atrophy[42].

This leads to the conclusion, that ‘the perfect’ wound dressing should combine the following 3 properties: reduction of bacterial contamination, removal of wound debris and the stimulation of the cellular wound healing process[33].

Medical honey: no longer so alternative

Honey can be considered as one of the oldest, or even the oldest wound dressing known by humans. Honey was used as wound dressing in ancient Egypt (>1500 BC), described by Hippocrates (460-377 BC) and used throughout the Middle Ages[43,44]. For centuries honey was used in traditional and folk medicine, based on medical empirical knowledge. Nowadays, the wound healing potential of honey and area of medical use is investigated and clinically proven for a broad range of wounds[45,46]. In chronic wounds a hyper-inflammatory microenvironment[47] and bacterial infection[34] dominate and a switch in this environment is necessary for wound healing. Honey offers a combination of antiseptic, antioxidative, immunemodulatory and wound cleaning properties. Honey's natural composition of high sugar content, low pH and special honey compounds impair bacterial growth[48]. These compounds are classes of oxidatives (hydrogen peroxide) and aldehydes (methylglyoxal[49,50], glyoxal[34]), as well as phenolic compounds[51] and bee-defensin-1[49,52], which is unique for honey. Different honeys show bactericidal activities against a broad range of bacteria[53]: against about 130 different multiple antibiotic resistances clinical isolates[54] and also against bacterial biofilms[55,56]. In addition, honey has a synergistic effect with antibiotics[57].

In wounds, honey acts as an immune-modulator, providing both pro- and anti-inflammatory properties. The stimulation of immune cells with honey leads to the release of pro-inflammatory cytokines as interleukin-6 (IL-6)[58,59], IL-1ß[59] and tumor necrosis factor alpha[59-62], whereas, honey also decreases reactive oxygen species production[60,63,64], respiratory burst activity[60], antibody production[64,65] and the concentration of inducible nitric oxide synthases[66], cyclooxygenase-2[66] prostaglandin E2[66-68] and nitric oxide release[66,69].

The antioxidant capacity of honeys results from the combined activity of compounds, as phenolic compounds, enzymes, peptides, organic acids, Maillard reaction products and other minor substances[70-73]. In addition, also a correlation was seen for total free amino acids and protein content[71], and honey can reduce the formation of lipid hydroperoxides and delays lipid oxidation[74,75]. Furthermore honey proved to facilitate wound debridement in chronic wounds[47].

In summary, honey possesses all characteristics to qualify as a wound healing agent, not only as an 'alternative', but also as a treatment of 'first choice'. After the first medical honey product (Medihoney) was registered by medical authorities and launched in 1999[76], nowadays one can find about 20 different FDA approved honey (containing) products[77], offering a novel antiseptic and wound healing treatment strategy for a broad range of chronic and difficultly healing wounds.

Outline of the thesis

Honey and other beehive products proved to be an effective treatment in a diverse range of diseases in otorhinolaryngology, as descripted in the narrative review in **chapter 2**. In **chapter 3** we showed that peroxide and non-peroxide honeys both have similar inhibitory effects on different strains of bacteria and qualify as good free radical scavengers, even at low concentration. We detected that the α-oxaldehydes methylglyoxal and glyoxal are strong antibacterial agents in honey, by the formation of different reactive oxygen species in reaction with hydrogen peroxide and the amino acids lysine and arginine. In **chapter 4** we focus on the histopathological origin of chronically discharging open mastoid cavities. We discovered that in patients with this chronic condition an open mastoid cavity is covered either with stratified squamous (keratinized) epithelium, respiratory columnar epithelium or granulation tissue. The typing of the cavity coverage is important for treatment expectations and we found that cavities covered with respiratory epithelium are less likely to heal, possibly by bacterial infection and local immunological factors. About 20% of patients with an open mastoid cavity suffer from chronic discharge, a condition, which often is therapy-resistant. We report the outcomes of a clinical randomized controlled trial in **chapter 5**, in which patients with a chronically infected open mastoid cavity, were treated with either conventional eardrops (Terra-Cortril Polymyxin B) or a medical honey gel (NasuMel). We proved that the treatment with honey gel is safe and decreased discomfort, otorrhea and inflammation and reduced bacterial infection in these patients. Another chronic ear condition is eczematous external otitis. In **chapter 6** we present the results of an observational study. We treated patients, who were suffering from recurrent eczematous external otitis with medical honey eardrops for 2 weeks. Treatment reduced discomfort and itching and clinical signs of eczema. In addition, we showed that these honey eardrops had a strong inhibitory activity against bacteria in vitro. In **chapter 7** we provide a general discussion and the future perspectives of the findings of this thesis, and give a short summary.

References

1. Celebi Erdivanli O, Kazikdas KC, Ozergin Coskun Z, Demirci M. Skin prick test reactivity in patients with chronic eczematous external otitis. Clinical and experimental otorhinolaryngology 2011; 4:174-176.
2. Nutten S. Atopic dermatitis: global epidemiology and risk factors. Ann Nutr Metab 2015; 66 Suppl 1:8-16.
3. Bos JD, Brenninkmeijer EE, Schram ME, Middelkamp-Hup MA, Spuls PI, Smitt JH. Atopic eczema or atopiform dermatitis. Experimental dermatology 2010; 19:325-331.
4. Sivaranjani N, Rao SV, Rajeev G. Role of reactive oxygen species and antioxidants in atopic dermatitis. Journal of clinical and diagnostic research : JCDR 2013; 7:2683-2685.
5. Birnie AJ, Bath-Hextall FJ, Ravenscroft JC, Williams HC. Interventions to reduce Staphylococcus aureus in the management of atopic eczema. Cochrane Database Syst Rev 2008:CD003871.
6. Wesemann DR, Nagler CR. The Microbiome, Timing, and Barrier Function in the Context of Allergic Disease. Immunity 2016; 44:728-738.
7. Yariktas M, Doner F, Dogru H, Demirci M. Asymptomatic food hypersensitivity prevalence in patients with eczematous external otitis. Am J Otolaryngol 2004; 25:1-4.
8. Yariktas M, Yildirim M, Doner F, Baysal V, Dogru H. Allergic contact dermatitis prevalence in patients with eczematous external otitis. Asian Pacific journal of allergy and immunology / launched by the Allergy and Immunology Society of Thailand 2004; 22:7-10.
9. Browning GG. The unsafeness of 'safe' ears. J Laryngol Otol 1984; 98:23-26.
10. Premachandra DJ, Woodward B, Milton CM, Sergeant RJ, Fabre JW. Treatment of chronic mastoiditis by grafting of mastoid cavities with autologous epithelial layers generated by in vitro culture of buccal epithelium. J Laryngol Otol 1991; 105:413-416.
11. Singh B, Maharaj TJ. Radical mastoidectomy: its place in otitic intracranial complications. J Laryngol Otol 1993; 107:1113-1118.
12. Youngs R. Epithelial migration in open mastoidectomy cavities. J Laryngol Otol 1995; 109:286-290.
13. Premachandra DJ, Woodward BM, Milton CM, Sergeant RJ, Fabre JW. Treatment of postoperative otorrhoea by grafting of mastoid cavities with cultured autologous epidermal cells. Lancet 1990; 335:365-367.
14. Beales PH. Complications following obliterative mastoid operations. Arch Otolaryngol 1969; 89:196-198.
15. Palva T. Surgical treatment of chronic middle ear disease. III. Revisions after tympanomastoid surgery. Acta Otolaryngol 1988; 105:82-89.
16. Youngs R. Temporal bone histopathology of open mastoidectomy cavities. J Laryngol Otol 1993; 107:569-573.
17. Jothiramalingam S, Kumar D, Kumar P, Sasindran V, Kumar N. Atticoantral disease — revisited. Indian Journal of Otolaryngology and Head & Neck Surgery 2007; 59:203-206.

18. Gluth MB, Tan BY, Santa Maria PL, Atlas MD. Unique microbiology of chronically unstable canal wall down tympanomastoid cavities: considerations for surgical revision. J Laryngol Otol 2013; 127:458-462.
19. Pata YS, Ozturk C, Akbas Y, Unal M, Gorur K, Ozcan C. Microbiology of cerumen in patients with recurrent otitis externa and cases with open mastoidectomy cavities. J Laryngol Otol 2004; 118:260-262.
20. Takoudes TG, Haddad J, Jr. Hydrogen peroxide in acute otitis media in guinea pigs. Laryngoscope 1997; 107:206-210.
21. Takoudes TG, Haddad J, Jr. Free radical production by antibiotic-killed bacteria in the guinea pig middle ear. Laryngoscope 2001; 111:283-289.
22. Somers T, Duinslaeger L, Delaey Bet al. Stimulation of epithelial healing in chronic postoperative otorrhea using lyophilized cultured keratinocyte lysates. Am J Otol 1997; 18:702-706.
23. Schilling JA. Wound healing. Surg Clin North Am 1976; 56:859-874.
24. Schreml S, Szeimies RM, Prantl L, Landthaler M, Babilas P. Wound healing in the 21st century. J Am Acad Dermatol 2010; 63:866-881.
25. Goldman R. Growth factors and chronic wound healing: past, present, and future. Adv Skin Wound Care 2004; 17:24-35.
26. Eming SA, Kaufmann J, Lohrer R, Krieg T. [Chronic wounds. Novel approaches in research and therapy]. Hautarzt 2007; 58:939-944.
27. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T 2015; 40:277-283.
28. The antibiotic alarm. Nature 2013; 495:141.
29. Piddock LJ. The crisis of no new antibiotics--what is the way forward? Lancet Infect Dis 2012; 12:249-253.
30. Norman G, Dumville JC, Mohapatra DP, Owens GL, Crosbie EJ. Antibiotics and antiseptics for surgical wounds healing by secondary intention. Cochrane Database Syst Rev 2016; 3:CD011712.
31. Sheldon AT, Jr. Antiseptic “resistance”: real or perceived threat? Clin Infect Dis 2005; 40:1650-1656.
32. Liptak JM. An overview of the topical management of wounds. Aust Vet J 1997; 75:408-413.
33. Atiyeh BS, Dibo SA, Hayek SN. Wound cleansing, topical antiseptics and wound healing. Int Wound J 2009; 6:420-430.
34. Roberts CD, Leaper DJ, Assadian O. The Role of Topical Antiseptic Agents Within Antimicrobial Stewardship Strategies for Prevention and Treatment of Surgical Site and Chronic Open Wound Infection. Adv Wound Care (New Rochelle) 2017; 6:63-71.
35. Khan MN, Naqvi AH. Antiseptics, iodine, povidone iodine and traumatic wound cleansing. J Tissue Viability 2006; 16:6-10.
36. Saunders JE, Raju RP, Boone JL, Hales NW, Berryhill WE. Antibiotic resistance and otomycosis in the draining ear: culture results by diagnosis. Am J Otolaryngol 2011; 32:470-476.
37. Saunders JE, Raju RP, Boone J, Berryhill W. Current bacteriology of suppurative otitis: resistant patterns and outcomes analysis. Otol Neurotol 2009; 30:339-343.

38. Sood S, Strachan DR, Tsikoudas A, Stables GI. Allergic otitis externa. Clinical otolaryngology and allied sciences 2002; 27:233-236.
39. Fraki JE, Kalimo K, Tuohimaa P, Aantaa E. Contact allergy to various components of topical preparations for treatment of external otitis. Acta Otolaryngol 1985; 100:414-418.
40. Millard TP, Orton DI. Changing patterns of contact allergy in chronic inflammatory ear disease. Contact dermatitis 2004; 50:83-86.
41. Devos SA, Mulder JJ, van der Valk PG. The relevance of positive patch test reactions in chronic otitis externa. Contact dermatitis 2000; 42:354-355.
42. Lubach D, Bensmann A, Bornemann U. Steroid-induced dermal atrophy. Investigations on discontinuous application. Dermatologica 1989; 179:67-72.
43. Forrest RD. Early history of wound treatment. J R Soc Med 1982; 75:198-205.
44. Pecanac M, Janjic Z, Komarcevic A, Pajic M, Dobanovacki D, Miskovic SS. Burns treatment in ancient times. Med Pregl 2013; 66:263-267.
45. Jull AB, Rodgers A, Walker N. Honey as a topical treatment for wounds. Cochrane Database Syst Rev 2008:CD005083.
46. Molan PC. The evidence supporting the use of honey as a wound dressing. Int J Low Extrem Wounds 2006; 5:40-54.
47. Shah A, Amini-Nik S. The Role of Phytochemicals in the Inflammatory Phase of Wound Healing. Int J Mol Sci 2017; 18.
48. Olaitan PB, Adeleke OE, Ola IO. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. Afr Health Sci 2007; 7:159-165.
49. Kwakman PH, te Velde AA, de Boer L, Speijer D, Vandenbroucke-Grauls CM, Zaat SA. How honey kills bacteria. Faseb J 2010; 24:2576-2582.
50. Mavric E, Wittmann S, Barth G, Henle T. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium) honeys from New Zealand. Mol Nutr Food Res 2008; 52:483-489.
51. Estevinho L, Pereira AP, Moreira L, Dias LG, Pereira E. Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. Food Chem Toxicol 2008; 46:3774-3779.
52. Kwakman PH, de Boer L, Ruyter-Spira CPet al. Medical-grade honey enriched with antimicrobial peptides has enhanced activity against antibiotic-resistant pathogens. Eur J Clin Microbiol Infect Dis 2011; 30:251-257.
53. Lu J, Carter DA, Turnbull Let al. The effect of New Zealand kanuka, manuka and clover honeys on bacterial growth dynamics and cellular morphology varies according to the species. PLoS One 2013; 8:e55898.
54. George NM, Cutting KF. Antibacterial Honey (Medihoney): in-vitro Activity Against Clinical Isolates of MRSA, VRE, and Other Multiresistant Gram-negative Organisms Including Pseudomonas aeruginosa. Wounds 2007; 19:231-236.
55. Majtan J, Bohova J, Horniackova M, Klaudiny J, Majtan V. Anti-biofilm effects of honey against wound pathogens Proteus mirabilis and Enterobacter cloacae. Phytother Res 2014; 28:69-75.
56. Alandejani T, Marsan J, Ferris W, Slinger R, Chan F. Effectiveness of honey on

Staphylococcus aureus and Pseudomonas aeruginosa biofilms. Otolaryngol Head Neck Surg 2009; 141:114-118.

57. Jenkins R, Cooper R. Improving antibiotic activity against wound pathogens with manuka honey in vitro. PLoS One 2012; 7:e45600.

58. Timm M, Bartelt S, Hansen EW. Immunomodulatory effects of honey cannot be distinguished from endotoxin. Cytokine 2008; 42:113-120.

59. Tonks AJ, Cooper RA, Jones KP, Blair S, Parton J, Tonks A. Honey stimulates inflammatory cytokine production from monocytes. Cytokine 2003; 21:242-247.

60. Tonks A, Cooper RA, Price AJ, Molan PC, Jones KP. Stimulation of TNF-alpha release in monocytes by honey. Cytokine 2001; 14:240-242.

61. Simuth J, Bilikova K, Kovacova E, Kuzmova Z, Schroder W. Immunochemical approach to detection of adulteration in honey: physiologically active royal jelly protein stimulating TNF-alpha release is a regular component of honey. J Agric Food Chem 2004; 52:2154-2158.

62. Tonks AJ, Dudley E, Porter NGet al. A 5.8-kDa component of manuka honey stimulates immune cells via TLR4. J Leukoc Biol 2007; 82:1147-1155.

63. Ahmad A, Khan RA, Mesaik MA. Anti inflammatory effect of natural honey on bovine thrombin-induced oxidative burst in phagocytes. Phytother Res 2009; 23:801-808.

64. Mesaik MA, Azim MK, Mohiuddin S. Honey modulates oxidative burst of professional phagocytes. Phytother Res 2008; 22:1404-1408.

65. Al-Waili NS. Effects of daily consumption of honey solution on hematological indices and blood levels of minerals and enzymes in normal individuals. J Med Food 2003; 6:135-140.

66. Raso GM, Meli R, Di Carlo G, Pacilio M, Di Carlo R. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. Life Sci 2001; 68:921-931.

67. Al-Waili NS, Boni NS. Natural honey lowers plasma prostaglandin concentrations in normal individuals. J Med Food 2003; 6:129-133.

68. Al-Waili NS. Effects of honey on the urinary total nitrite and prostaglandins concentration. Int Urol Nephrol 2005; 37:107-111.

69. Kassim M, Achoui M, Mustafa MR, Mohd MA, Yusoff KM. Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. Nutr Res 2010; 30:650-659.

70. Gheldof N, Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. J Agric Food Chem 2002; 50:3050-3055.

71. Perez RA, Iglesias MT, Pueyo E, Gonzalez M, de Lorenzo C. Amino acid composition and antioxidant capacity of Spanish honeys. J Agric Food Chem 2007; 55:360-365.

72. Alvarez-Suarez JM, Gonzalez-Paramas AM, Santos-Buelga C, Battino M. Antioxidant characterization of native monofloral Cuban honeys. J Agric Food Chem 2010; 58:9817-9824.

73. Al-Mamary M A-MA, Al-Habori M. . Antioxidant activities and total phenolics of different types of honey. . Nutr Res 2002; 22:1041–1047.
74. Johnston JE, Sepe HA, Miano CL, Brannan RG, Alderton AL. Honey inhibits lipid oxidation in ready-to-eat ground beef patties. Meat Science 2005; 70:627-631.
75. Subrahmanyam M, Shahapure A, Nagane N. Free radical control—the main mechanism of the action of honey in burns. . Ann Burns Fire Disasters 2003; 16:135-138.
76. Molan P. Use of honey in wound care by medical professionals. Periodical [serial online]. Date 2012. Available from, http://www.academia.edu.
77. Saikaly SK, Khachemoune A. Honey and Wound Healing: An Update. American journal of clinical dermatology 2017; 18:237-251.

CHAPTER 2

-

Honey and beehive products in otorhinolaryngology: a narrative review

Darius Henatsch, Frederik Wesseling, Kenneth W. Kross, Robert J. Stokroos

Clinical Otolaryngology. 2016 Oct;41(5):519-531

Abstract

Background: Honey and beehive products were rediscovered as an alternative treatment in wounds. The medicinal properties also raised interest of their use in otorhinolaryngology.

Objective of review: To give an overview of the effectiveness of beehive products in otorhinolaryngology.

Type of review: Narrative.

Search strategy and evaluation: A literature search of the databases PubMed, EMBASE and Cochrane was performed from the last 2 decades till December 2014. The search terms 'honey', 'propolis' or 'royal jelly' were used. Articles, which evaluated the effectiveness of beehive products in otorhinolaryngology, were included. The quality assessment of included studies was performed using the Cochrane Collaboration's risk of bias tool.

Discussion and conclusion: A total of 36 studies were identified and evaluated. Eighteen studies investigated their effect in oral infections, seven in infection of the respiratory tract, six in rhino-sinusal diseases, four investigated the use in tonsillectomy and head and neck surgery and one study explored the preventive effect in otitis media. Honey can be considered as effective (additional) treatment in mucositis, childhood cough, persistent post-infectious cough and after tonsillectomy. Propolis may have a role in the treatment of (aphtous) stomatitis, mouth ulcer and prevention of acute otitis media. Royal jelly showed to reduce mucositis. In the presented studies, beehive products proved to be safe, with only minor adverse reactions. Studies showed to be diverse and had some methodological limitations.

Introduction

Beehive products, such as honey and propolis, have been mentioned as wound dressings in ancient writings from Egypt and Greece[1,2]. Honey is a by-product of flower nectar produced in the aero-digestive tract of bees, and propolis is produced from plant resins, enriched with salivary enzymatic secretions[2,3]. Honey and propolis are complex substances and contain about 180 different substances[2,4]. While honey contains mainly fructose and glucose and minor components such as organic acids, vitamins, flavonoids and enzymes[4-6], propolis contains polyphenols, volatile oils, aromatic acids and waxes[2]. Another less common beehive product is royal jelly (RJ). This substance is secreted from the hypopharyngeal and mandibular glands of worker bees as nutrition for the queen and larvae[7]. RJ is rich in carbohydrates, lipids, vitamins, and proteins[8].

Beehive products are nowadays 're-discovered' in wound healing. The clinical effectiveness of honey in the treatment of different kinds of wounds is well established[9]. Honey leads to faster epithelization, wound area reduction and less inflammation[10]. The unique composition leads to three major convalescence stimulating functions; honey[11-16], propolis[17] and RJ[18,19] have immune-modulatory and antimicrobial capacities[7,20-23]. Honey and propolis have in addition strong antioxidative properties, caused by phenolic compounds and amino acids[24-29]. The increasing awareness of beehive products as alternative wound treatment generated interest of their use in otorhinolaryngology[30], with an increasing number of publications referring these products[31-33]. The aim of this study is to describe and evaluate all studies investigating the possible beneficial effects of beehive products in otorhinolaryngology, which has not been provided so far.

Methods

A search of all available literature was performed, using the online databases PubMed, EMBASE and the Cochrane Central Register of Controlled Trials. Studies were identified using the search terms: *honey, propolis* or *royal jelly* independently. The search was restricted to human species and articles published in English, German, French or Dutch from the last 2 decades till December 2014. Two authors independently screened ref erence lists of relevant reviews and included articles for additional literature. Trials that were randomized controlled trials, prospective or retrospective cohort studies, in which beehive products were used as prevention or therapy of diseases in otorhinolaryngology, were included in this review. First, titles and abstracts of articles of databases were screened and identified for eligibility with a removal of duplicates. After screening, selected articles were evaluated independently by two reviewers. Case series, editorials, letters, comments and guidelines were excluded. After selection of studies, we used the Cochrane Collaboration's tool for assessing risk of bias[34]. All trials were rated for risk of bias, depending on the description of sequence generation, allocation concealment, blinding, selective outcome reporting and incomplete data.

Quality assessment of included studies

The studies discussed in the presented review had methodological limitations. Only five of the 36 included trials were assessed as being at overall low risk of bias[35-39]. Eleven were assessed as being at unclear risk of bias[40-50], and all remaining studies were assessed as being at overall high risk of bias. Details on quality assessment are presented in Fig. 1.

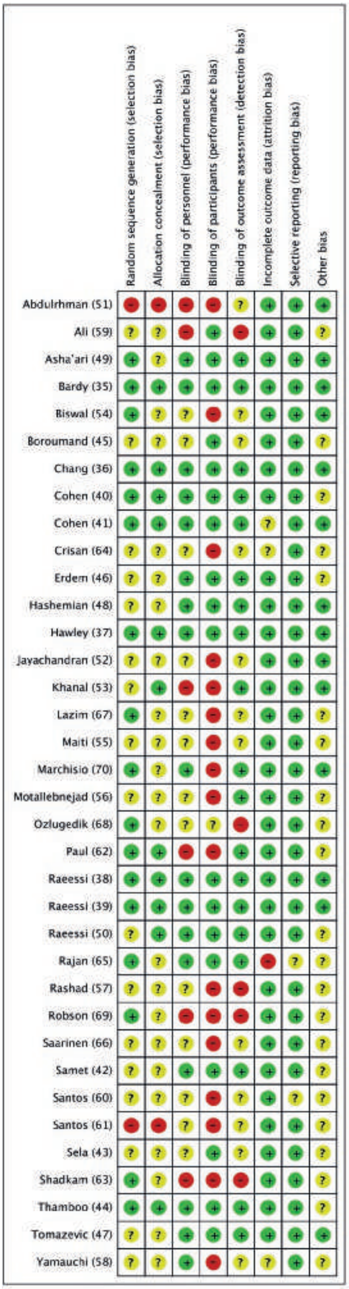


Fig. 1: Risk of bias summary.

Results and discussion

A total of 3850 abstracts were reviewed of which 36 full-text articles were identified. All three different beehive products are used for the prevention and therapy of various conditions in otorhinolaryngology. Eighteen studies investigated their effect in oral infections, seven in infection of the respiratory tract, six in rhino-sinusal diseases, four investigated the use in tonsillectomy and head and neck surgery and one study explored the preventive effect in otitis media. Study characteristics are shown in Table 1.

Oral infection

Half of all studies included in this review investigated the effect of honey, propolis or RJ in oral pathologies. In patients who underwent radiotherapy[52-54,56], with concomitant chemotherapy[55,57] of the oropharyngeal region, honey treatment resulted in less severe[52-57] oral mucositis, with later onset[52,55], faster healing[52] and a reduction in candida infection and pathogenic bacteria[57]. In patients with already existing oral mucositis, honey treatment upon chemotherapy led to less severe mucositis, with the highest effect for honey plus caffeine treatment[39]. In chemoradiation-induced mucositis, RJ as therapy additional to mouthwash resulted in faster mucositis healing compared to mouthwash solely[46] and had a preventive effect on mucositis onset, severity and healing[58].

A similar effect was seen in children with chemotherapy-induced oral mucositis, where honey treatment showed faster complete recovery time and healing, without an influence on severity and onset of mucositis[51]. In a similar study, propolis was not superior to placebo in preventing severe mucositis in children[47]. Two studies found no differences in mucositis incidence between honey and placebo treatment during radiation therapy[35,37]. In addition, honey led to decreased streptococcus mutans counts in patients with radiation-induced xerostomia[43].

The treatment of patients with recurrent aphtous stomatitis with a propolis capsule, reduced outbreak frequency and improved quality of life[42]. Furthermore, propolis products had the same effect compared to local antifungal medication in patients with denture stomatitis[61] or denture-wearing patients with oral candidiasis[60] and enhanced ulcer healing with less pain in patients with mouth ulcers[59].

The antioxidant capacity of honey and propolis could explain the positive study results[24-27,29,71]. They act as a scavenger of reactive oxygen species, which are an inevitable by-product of anti-cancer therapies and a cause of mucositis[72,73]. The antioxidant capacities are comparable to agents, as Zinc sulphate or amifostine[74].

In addition, honey[75-77] and propolis[22] have a strong antibacterial capacity without bacterial resistance formation[78,79] and can eradicate bacteria even in the form of biofilms[80].

Table 1 Study characteristics

Source	No. of pat/cont	Indication	Test, control	Outcomes	Follow-up	Adverse reaction
Abdulrhman[51]	30/30/30	chemotherapy induced oral mucositis	natural honey (Egypt), HOPE (honey, olive oil–propolis extract, beeswax), benzocaine gel	recovery time to complete healing	ca 10 d	burning mouth sensation (n=8)
Raeessi[39]	23/23/23	chemotherapy induced oral mucositis	honey (west Iran), coffee/honey, betamethasone	mucositis grading/VAS	2 w	not mentioned
Tomazevic[47]	26/24	chemotherapy induced oral mucositis	Chinese propolis (ethanolic extract), placebo	mucositis grading/duration	during chemo (<6 m)	none
Hawley[37]	54/52	radiation induced oral mucositis	Manuka honey (New Zeeland), placebo	mucositis grade	7 days after radiation	burning sensation (n=2)
Jayachandran[52]	20/20/20	radiation induced oral mucositis	Dabur honey, Benzydamine hydrochloride, normal saline	mucositis grade	2 weeks after radiation	not mentioned
Khanal[53]	20/20	radiation induced oral mucositis	natural honey (India), lignocaine gel	mucositis grade	6 w	not mentioned
Bardy[35]	66/63	radiation induced oral mucositis	Manuka honey (New Zealand), placebo	development of high grade mucositis, microbial colonization, clinical presentation	42 d	not mentioned
Biswal[54]	20/20	radiation induced oral mucositis	natural honey (Malaysia), no treatment	mucositis grade, change in body weight	7 w	not mentioned
Erdem[46]	51/52	radio-chemotherapy induced oral mucositis	mouthwash with royal jelly, mouthwash	recovery time to complete healing	till complete healing	none
Maiti[55]	30/30	radio-chemotherapy induced oral mucositis	natural honey, no treatment	mucositis grade, fasting glucose, body weight	6 w after radiation	not mentioned

Motallebnejad[56]	20/20	radiation induced oral mucositis	natural honey (Iran), normal saline	mucositis grade, change in body weight	6 w	not mentioned
Rashad[57]	20/20	radio-chemotherapy induced oral mucositis	natural honey (Clover plant), no treatment	mucositis grade, microbiological colonization	6 w	not mentioned
Yamauchi[58]	7/6	radio-chemotherapy induced mucositis	Chinese propolis, no treatment	mucositis grading	1 m after radiation	not mentioned
Sela[43]	12/12	radiation induced xerostomia, normal volunteers	wildflower honey (Israel)	bacteria count	5 min	not mentioned
Ali[59]	40/40/40	mouth ulcers	propolis paste (with either olive or sesame oil), placebo	pain, ulcer complete healing, size reduction	1 w	none
Samet[42]	10/9	recurrent aphtous stomatitis	bee propolis (Vitamin World), calcium-based food supplement	aphthous ulcers (duration and severity)	6 - 13 m	not mentioned
Santos[60]	12/6	oral candidiasis	propolis extract (Brazil), Micostatin/Nystatin	mucosal evaluation, infection	7 d	not mentioned
Santos[61]	15/15	denture stomatitis	propolis gel (Brazil), Miconazol gel	palatal erythema	7 d	none
Cohen[41]	75/75/75/75	nocturnal cough attributed to URTI	eucalyptus-, labiatae-, citrus-honey, Silan date extract	cough (frequence, severity, bothersome), quality of sleep of children and parents	1 d	stomachache, nausea, vomiting (n=4)
Paul[62]	130	nocturnal cough attributed to URTI	buckwheat honey, honey flavored Dextromethorphan, no treatment	cough (frequence, severity, bothersome), quality of sleep of children and parents	1 d	hyperactivity, nervousness, insomnia (n=5), drowsiness (n=1), stomachache, nausea, vomiting (n=2)

Shadkam[63]	40/40/40/40	nocturnal cough attributed to URTI	natural honey (Iran), Dextromethorphan, Diphenhydramine, supportive treatment	cough (frequence, severity), quality of sleep of children and parents	1 d	nervousness (n=2)
Raeessi[50]	16/14/54	persistent postinfectious cough	honey (west Iran), coffee, coffee/honey	frequency of cough	9 w	not mentioned
Raeessi[38]	29/30/26	persistent postinfectious cough	coffee/honey (west Iran), prednisolone, placebo	frequency of cough	2 w	not mentioned
Cohen[40]	215/215	prevention of URTI	Chizukit (propolis, Echinacea, vitamin C), placebo	episodes of URTI, symptoms of illness, medication use, absence from day care, physician visits	12 w	mild gastrointestinal and palatability symptoms (n=9)
Crisan[64]	61/33	prevention of URTI	NIVCRISOL (propolis extract), normal saline	Clinical signs of rhinopharingitis, disease of the tracheobronchial tree, nasopharyngeal infection	5 m	none
Chang[36]	16/16/16 o.c	chronic or allergic fungal rhinosinusitis	Manuka honey (New Zeeland), gentamicin, budesonide, normal saline	discomfort, pain, histologic grading (inflammation/ healing)	28 d	none
Hashemian[48]	32/32	chronic rhinosinusitis	thyme honey, placebo	SNOT-22, CT scan, endoscopy scoring	60 d	burning sensation (no difference from control group)
Asha'ari[49]	20/20	allergic rhinitis	Tualang honey (Malaysia), placebo	symptoms of allergic rhinitis	8 w	not mentioned
Rajan[65]	12/12/13	allergic rhinoconjunctivitis	natural honey (England), Clover honey (England), placebo	upper respiratory and ocular symptoms, use of anti-allergic medication	30 w	not mentioned
Thamboo[44]	38 o.c	allergic fungal rhinosinusitis	honey (saline solution, New Zealand), no treatment	endoscopic mucosal score, bacterial flora, questionnaire	28 d	nausea (n=1), burning sensation of the nasal mucosa (n=4)

Saarinen[66]	25/19/17	birch pollen allergy	natural honey (Finland), honey enriched with birch and alder pollen, no treatment	days with allergic symptoms (conjunctival, nasal, other), frequencies of days with medication	6 m	mild itching in the mouth or skin, runny nose (n=n.a)
Lazim[67]	63	tonsillectomy	Tualang honey (Malaysia), no treatment	healing process of the tonsillar fossa	14 d	adverse events all related to tonsillectomy
Boroumand [45]	52/52	(adeno)tonsillectomy	honey, placebo	VAS, number of painkiller, awakening at night due to pain, tonsillary fossa recovery	5 d	none
Ozlugedik[68]	30/30	(adeno)tonsillectomy	flower honey, placebo	pain, awakenings, tonsillary fossa recovery, medication use	14 d	none
Robson[69]	25/24	microvascular free tissue reconstruction	Manuka honey (New Zealand), no treatment	wound infection, hospital stay, satisfaction	28 d	none
Marchisio[70]	61/61	prevention of AOM	propolis extract (with zinc sulfate), no treatment	AOM, febrile respiratory illnesses, medication use, nasopharyngeal colonization	3 m	rash (n=1), vomiting (n=1)

Abbreviations: AOM, acute otitis media; cont, control; d, days; m, minutes; n.a, not available; o.c, own control; pat, patient; URTI, upper respiratory tract infection; w, weeks, y, years

2

Upper respiratory tract

Three studies evaluated the effect of honey consumption on nocturnal childhood cough. Single dose treatment with honey was superior to over-the-counter cough medication[62,63] or silan date extract[41]. With regard to the prevention of respiratory tract illness in children, Echinacea-propolis-vitamin C preparation showed a positive effect after 12 weeks of treatment[40]. In the latter study it is difficult to prove the effect of solely propolis, as Echinacea and vitamin C may play a role in the prevention of common cold in children[81,82]. Moreover, the nasal application of a propolis extract was evaluated in children. After 5 months treatment, less disease episodes were observed without difference for rhinorrhea[64]. Based on these outcomes and contradictable results of over-the-counter antitussive medication[83], honey can be seen as a potential alternative treatment of cough and illness of the upper respiratory tract in children.

In adults, the treatment of persistent post-infectious cough with honey combined with caffeine seemed to be more effective than systemic steroids[38]. The effect of honey was enhanced by the addition of caffeine[50].

Rhino-sinusal diseases

The beneficial effects of honey administration in allergic fungal rhinosinusitis (AFRS), chronic rhinosinusitis and allergic rhinitis were evaluated. Honey did not show to improve sinonasal mucosal healing after Merocel packing after functional endoscopic sinus surgery (FESS) in patients suffering from ARFS[36], but showed a symptom improvement without a clear mucosal and bacteriological enhancement in ARFS patients[44]. In patients with bilateral chronic rhinosinusitis undergoing FESS, no changes in symptoms, endoscopy and CT scan were observed after nasal honey spray application[48].

Furthermore, honey ingestion had no effect in the treatment of allergic rhinoconjunctivitis[65] and only led to an improvement of individual allergic symptoms but did not affect total symptom score in allergic rhinitis[49]. However, birch pollen-enriched honey led to an individual symptom reduction in allergic patients66. These effects can partly be contributed to the immune-modulatory capacities of honey, which were demonstrated in several *in vitro* studies[15,16,84].

Wound healing

Intra-operative administration and postoperative consumption of honey after adeno-tonsillectomy[45,68] or tonsillectomy[67] in children led to VAS improvement, less use of painkillers[45,68] and faster tonsillary fossa healing[67,68]. In patients who required micro-vascular-free tissue reconstruction after the operation of head and neck cancer, honey dressing did not lead to less wound site infection. However, a shorter hospital stay was observed[69]. There are conflicting results about the role of honey in acute skin wound healing[9,85], but honey can be seen as a good alternative postoperative therapy after tonsillectomy compared to perioperative coatings or topical anesthetics, which seemed to be ineffective so far[86-90].

Otitis media

One study investigated the effect of propolis in otological disease[70]. Herein, the treatment with a propolis-zinc suspension resulted in less acute otitis media (AOM) episodes and a decrease of nasopharyngeal colonization in children with a history of recurrent AOM. Whether this effect is attributed to propolis alone cannot be concluded from this study. Zinc itself is known to reduce common cold symptoms in healthy people[91] and may have a possible role in the incidence reduction of otitis media[92].

In this review, we present the results of 36 clinical studies, which evaluate the effects of beehive products in otorhinolaryngology. The effect of interventions is summarized in Table 2. Off all studies, 56% reported the occurrence of adverse events. In 28% of these studies no adverse events were observed at all. The other studies reported only mild events in a low percentage of participants, mostly mild gastrointestinal and palatability symptoms, mild itching and rash in the mouth or skin and hyperactivity and nervousness in children. Importantly, due to the appearance of Clostridium botulinum spores in honey, any honey treatment of children during the first year of life is discouraged due to the risk of infant botulism, a rare but life-threatening disease[93,94].

In the studies, a broad diversity of sorts of mainly honey and propolis was used, so it is not possible to recommend one single product, knowing that composition and properties can differ by factors as environmental conditions and exudates[5]. For further research, it is strongly recommended to standardize type and preparation of beehive products for a better understanding of their properties and efficacy. Furthermore, this review highlights the importance and the present lack of high quality clinical trials, investigating the potential effects of beehive products in otorhinolaryngology.

Table 2. Effect of beehive products in otorhinolaryngology

symptom	intervention	type of trial	overall risk of bias	main outcome	harms/adverse reaction	comment
Mucositis[35,37,39,51-57]	honey	9 RCTs n=40-131 1 RCT n=90 in children	high (randomization, blinding)	less severe mucositis, later onset and faster healing	only mentioned in 2 trials: burning mouth sensation (n=2)	likely beneficial in adults, unclear effect in children
Mucositis[46,58]	RJ	2 RCTs n=13, n=103	moderate to high (blinding, randomization)	less severe mucositis with faster healing	none / not mentioned	likely beneficial
Mucositis[47]	propolis	single RCT n=50	moderate (randomization)	children: no benefit in severe mucositis	none	no benefit in children
Recurrent aphtous stomatitis[42]	propolis	single RCT n=19	moderate (randomization)	reduction of outbreak, better quality of life	not mentioned	probable benefit
Candidiasis[60]	propolis	single RCT n=18	high (blinding, randomization, other)	complete remission in treatment and control group, no placebo treatment	not mentioned	unclear (likely no) benefit
Denture stomatitis[61]	propolis	single RCT n=30	high (blinding, randomization)	complete remission in treatment and control group, no placebo treatment	none	unclear (likely no) benefit
Mouth ulcer[59]	propolis	single RCT n=120	high (blinding, randomization)	less pain, faster healing	none	probable benefit

Radiation induced xerostomia[43]	honey	single trial n=24	no RCT, comparison with healthy volunteers	decrease of Streptococcus mutans count but not total bacteria	not mentioned	No clinically meaningful benefit
Nocturnal childhood cough [41,62,63]	honey	3 RCTs n=130-300	low in one trial with 300 children, high in other trials (no blinding)	less nocturnal cough with enhancement of quality of sleep of children and parents	hyperactivity, nervousness, insomnia (n=7), drowsiness (n=1), stmachache, nausea, vomiting (n=6)	likely beneficial in children (single dose treatment for one night)
Persistent postinfectious cough[38,50]	honey (honey with coffee in 1 trial)	2 RCTs n=84, n=97	low to moderate (randomization not described)	decrease in frequency of cough	not mentioned	probable benefit
Respiratory tract infections in children[40,64]	propolis, propolis solution (with Echinacea, vitamin c)	2 RCTs n=94, n=430	low and high (blinding, randomization)	less and shorter respiratory tract infections	mild gastrointestinal and palatability symptoms (n=9)	unclear benefit, propolis applied as mixture and as nasal application
Rhinosinosal disease[36,44,48]	honey	3 RCT n=38-64 (own control in 2 trials)	low to moderate (other bias)	no improvement of discomfort after Merocel re-movement or on CT scan. Inconsistent results of endoscopic improvement and symptoms	nausea (n=1), burning sensation of the nasal mucosa (n=4)	unclear (likely no) benefit
Allergic rhinitis[49,65,66]	honey	3 RCT n=37-61	moderate to high (randomization, blinding drop-out, other)	inconsistent results of allergic rhinitis symptoms	mild itching in the mouth or skin, runny nose (n=n.a), not mentions in 2 trials	unclear (likely no) benefit

2

(Adeno-) tonsillectomy[45,67,68]	honey	3 RCT n=60-104	moderate to high (randomization, blinding, other)	less pain with faster recovery of tonsillary fossa	none	likely beneficial
microvascular free tissue reconstruction[69]	honey	single RCT n=49	high (randomization, blinding, other)	shorter duration of hospital stay	none	unclear (likely no) benefit
Otitis media[70]	propolis (+zinc suspension)	RCT n=122	high (randomization, blinding)	less occurrence of AOM and respiratory pathogens	rash (n=1), vomiting (n=1)	probable benefit

Conclusion

Honey only, or as add-on (alternative) therapy, has a possible beneficial effect in the treatment of mucositis, childhood cough, persistent post-infectious cough, as well as post-tonsillectomy wound healing and the prevention of AOM. Propolis has a potential role in the treatment of (aphtous) stomatitis and mouth ulcers, and RJ seemed to effectively reduce mucositis. In the presented studies, beehive products proved to be safe, with only minor adverse reactions. The standardization of mainly honey and propolis for further research is strongly recommended.

References

1. Lusby PE, Coombes A, Wilkinson JM. Honey: a potent agent for wound healing? J Wound Ostomy Continence Nurs 2002; 29:295-300.
2. Castaldo S, Capasso F. Propolis, an old remedy used in modern medicine. Fitoterapia 2002; 73 Suppl 1:S1-6.
3. Khalil ML. Biological activity of bee propolis in health and disease. Asian Pac J Cancer Prev 2006; 7:22-31.
4. Gheldof N, Wang XH, Engeseth NJ. Identification and quantification of antioxidant components of honeys from various floral sources. J Agric Food Chem 2002; 50:5870-5877.
5. Viuda-Martos M, Ruiz-Navajas Y, Fernandez-Lopez J, Perez-Alvarez JA. Functional properties of honey, propolis, and royal jelly. J Food Sci 2008; 73:R117-124.
6. Olaitan PB, Adeleke OE, Ola IO. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. Afr Health Sci 2007; 7:159-165.
7. Cherniack EP. Bugs as drugs, Part 1: Insects: the “new” alternative medicine for the 21st century? Alternative medicine review : a journal of clinical therapeutic 2010; 15:124-135.
8. Li X, Huang C, Xue Y. Contribution of lipids in honeybee (Apis mellifera) royal jelly to health. J Med Food 2013; 16:96-102.
9. Jull AB, Rodgers A, Walker N. Honey as a topical treatment for wounds. Cochrane Database Syst Rev 2008:CD005083.
10. Molan PC. The evidence supporting the use of honey as a wound dressing. Int J Low Extrem Wounds 2006; 5:40-54.
11. Al-Waili NS, Boni NS. Natural honey lowers plasma prostaglandin concentrations in normal individuals. J Med Food 2003; 6:129-133.
12. Al-Waili NS. Effects of honey on the urinary total nitrite and prostaglandins concentration. Int Urol Nephrol 2005; 37:107-111.
13. Al-Waili NS. Effects of daily consumption of honey solution on hematological indices and blood levels of minerals and enzymes in normal individuals. J Med Food 2003; 6:135-140.
14. Al-Waili NS, Boni NS. Honey increased saliva, plasma, and urine content of total nitrite concentrations in normal individuals. J Med Food 2004; 7:377-380.
15. Timm M, Bartelt S, Hansen EW. Immunomodulatory effects of honey cannot be distinguished from endotoxin. Cytokine 2008; 42:113-120.
16. Tonks AJ, Cooper RA, Jones KP, Blair S, Parton J, Tonks A. Honey stimulates inflammatory cytokine production from monocytes. Cytokine 2003; 21:242-247.
17. Sforcin JM. Propolis and the immune system: a review. J Ethnopharmacol 2007; 113:1-14.
18. Oka H, Emori Y, Kobayashi N, Hayashi Y, Nomoto K. Suppression of allergic reactions by royal jelly in association with the restoration of macrophage function and the improvement of Th1/Th2 cell responses. Int Immunopharmacol 2001; 1:521-532.
19. Kohno K, Okamoto I, Sano Oet al. Royal jelly inhibits the production of

proinflammatory cytokines by activated macrophages. Biosci Biotechnol Biochem 2004; 68:138-145.
20. Basualdo C, Sgroy V, Finola MS, Marioli JM. Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. Veterinary microbiology 2007; 124:375-381.
21. Lusby PE, Coombes AL, Wilkinson JM. Bactericidal activity of different honeys against pathogenic bacteria. Arch Med Res 2005; 36:464-467.
22. Silici S, Kutluca S. Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. J Ethnopharmacol 2005; 99:69-73.
23. Boukraa L. Additive activity of royal jelly and honey against Pseudomonas aeruginosa. Alternative medicine review : a journal of clinical therapeutic 2008; 13:330-333.
24. Perez RA, Iglesias MT, Pueyo E, Gonzalez M, de Lorenzo C. Amino acid composition and antioxidant capacity of Spanish honeys. J Agric Food Chem 2007; 55:360-365.
25. Alvarez-Suarez JM, Gonzalez-Paramas AM, Santos-Buelga C, Battino M. Antioxidant characterization of native monofloral Cuban honeys. J Agric Food Chem 2010; 58:9817-9824.
26. Gheldof N, Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. J Agric Food Chem 2002; 50:3050-3055.
27. Al-Mamary M A-MA, Al-Habori M. . Antioxidant activities and total phenolics of different types of honey. . Nutr Res 2002; 22:1041–1047.
28. Borrelli F, Maffia P, Pinto Let al. Phytochemical compounds involved in the anti-inflammatory effect of propolis extract. Fitoterapia 2002; 73 Suppl 1:S53-63.
29. Kumazawa S, Ahn MR, Fujimoto T, Kato M. Radical-scavenging activity and phenolic constituents of propolis from different regions of Argentina. Nat Prod Res 2010; 24:804-812.
30. Werner A, Laccourreye O. Honey in otorhinolaryngology: when, why and how? European annals of otorhinolaryngology, head and neck diseases 2011; 128:133-137.
31. Oduwole O, Meremikwu MM, Oyo-Ita A, Udoh EE. Honey for acute cough in children. Cochrane Database Syst Rev 2012; 3:CD007094.
32. Bardy J, Slevin NJ, Mais KL, Molassiotis A. A systematic review of honey uses and its potential value within oncology care. Journal of clinical nursing 2008; 17:2604-2623.
33. Worthington HV, Clarkson JE, Bryan Get al. Interventions for preventing oral mucositis for patients with cancer receiving treatment. Cochrane Database Syst Rev 2011:CD000978.
34. Review Manager (RevMan), Version 5.1 [computer program]: København, Denmark: Nordic Cochrane Centre 2011.
35. Bardy J, Molassiotis A, Ryder WDet al. A double-blind, placebo-controlled, randomised trial of active manuka honey and standard oral care for radiation-induced oral mucositis. Br J Oral Maxillofac Surg 2012; 50:221-226.

36. Chang EH, Alandejani T, Akbari E, Ostry A, Javer A. Double-Blinded, Randomized, Controlled Trial of Medicated versus Nonmedicated Merocel Sponges for Functional Endoscopic Sinus Surgery. J Otolaryngol-Head N 2011; 40:S14-S19.
37. Hawley P, Hovan A, McGahan CE, Saunders D. A randomized placebo-controlled trial of manuka honey for radiation-induced oral mucositis. Support Care Cancer 2014; 22:751-761.
38. Raeessi MA, Aslani J, Raeessi N, Gharaie H, Karimi Zarchi AA, Raeessi F. Honey plus coffee versus systemic steroid in the treatment of persistent post-infectious cough: a randomised controlled trial. Primary care respiratory journal : journal of the General Practice Airways Group 2013; 22:325-330.
39. Raeessi MA, Raeessi N, Panahi Yet al. "Coffee plus honey" versus "topical steroid" in the treatment of chemotherapy-induced oral mucositis: a randomised controlled trial. BMC Complement Altern Med 2014; 14:293.
40. Cohen HA, Varsano I, Kahan E, Sarrell EM, Uziel Y. Effectiveness of an herbal preparation containing echinacea, propolis, and vitamin C in preventing respiratory tract infections in children: a randomized, double-blind, placebo-controlled, multicenter study. Arch Pediatr Adolesc Med 2004; 158:217-221.
41. Cohen HA, Rozen J, Kristal Het al. Effect of honey on nocturnal cough and sleep quality: a double-blind, randomized, placebo-controlled study. Pediatrics 2012; 130:465-471.
42. Samet N, Laurent C, Susarla SM, Samet-Rubinsteen N. The effect of bee propolis on recurrent aphthous stomatitis: a pilot study. Clinical oral investigations 2007; 11:143-147.
43. Sela M, Maroz D, Gedalia I. Streptococcus mutans in saliva of normal subjects and neck and head irradiated cancer subjects after consumption of honey. J Oral Rehabil 2000; 27:269-270.
44. Thamboo A, Philpott C, Javer A, Clark A. Single-blind study of manuka honey in allergic fungal rhinosinusitis. Journal of otolaryngology - head & neck surgery = Le Journal d'oto-rhino-laryngologie et de chirurgie cervico-faciale 2011; 40:238-243.
45. Boroumand P, Zamani MM, Saeedi M, Rouhbakhshfar O, Hosseini Motlagh SR, Aarabi Moghaddam F. Post tonsillectomy pain: can honey reduce the analgesic requirements? Anesthesiology and pain medicine 2013; 3:198-202.
46. Erdem O, Gungormus Z. The effect of royal jelly on oral mucositis in patients undergoing radiotherapy and chemotherapy. Holistic nursing practice 2014; 28:242-246.
47. Tomazevic T, Jazbec J. A double blind randomised placebo controlled study of propolis (bee glue) effectiveness in the treatment of severe oral mucositis in chemotherapy treated children. Complementary therapies in medicine 2013; 21:306-312.
48. Hashemian F, Baghbanian N, Majd Z, Rouini MR, Jahanshahi J, Hashemian F. The effect of thyme honey nasal spray on chronic rhinosinusitis: a double-blind randomized controlled clinical trial. Eur Arch Otorhinolaryngol 2015; 272:1429-1435.
49. Asha'ari ZA, Ahmad MZ, Jihan WS, Che CM, Leman I. Ingestion of honey improves the symptoms of allergic rhinitis: evidence from a randomized placebo-controlled trial in the East coast of Peninsular Malaysia. Annals of Saudi medicine 2013; 33:469-475.

50. Raeessi MA, Aslani J, Gharaie H, Karimi Zarchi AA, Raeessi N, Assari S. Honey with Coffee: A new finding in the treatment of Persistent Postinfectious Cough. Iran J Otorhinolaryngol 2011; 23:1-8.

51. Abdulrhman M, El Barbary NS, Ahmed Amin D, Saeid Ebrahim R. Honey and a mixture of honey, beeswax, and olive oil-propolis extract in treatment of chemotherapy-induced oral mucositis: a randomized controlled pilot study. Pediatric hematology and oncology 2012; 29:285-292.

52. Jayachandran S, Balaji N. Evaluating the effectiveness of topical application of natural honey and benzydamine hydrochloride in the management of radiation mucositis. Indian journal of palliative care 2012; 18:190-195.

53. Khanal B, Baliga M, Uppal N. Effect of topical honey on limitation of radiation-induced oral mucositis: an intervention study. Int J Oral Maxillofac Surg 2010; 39:1181-1185.

54. Biswal BM, Zakaria A, Ahmad NM. Topical application of honey in the management of radiation mucositis. A Preliminary study. Support Care Cancer 2003; 11:242-248.

55. Maiti PK, Ray A, Mitra TN, Jana U, Bhattacharya J, Ganguly S. The effect of honey on mucositis induced by chemoradiation in head and neck cancer. J Indian Med Assoc 2012; 110:453-456.

56. Motallebnejad M, Akram S, Moghadamnia A, Moulana Z, Omidi S. The effect of topical application of pure honey on radiation-induced mucositis: a randomized clinical trial. J Contemp Dent Pract 2008; 9:40-47.

57. Rashad UM, Al-Gezawy SM, El-Gezawy E, Azzaz AN. Honey as topical prophylaxis against radiochemotherapy-induced mucositis in head and neck cancer. J Laryngol Otol 2009; 123:223-228.

58. Yamauchi K, Kogashiwa Y, Moro Y, Kohno N. The effect of topical application of royal jelly on chemoradiotherapy-induced mucositis in head and neck cancer: a preliminary study. International journal of otolaryngology 2014; 2014:974967.

59. Ali HS, Abdul Rasool BK. Propolis Buccal Paste in Treatment of Aphtous Ulceration: Formulation and Clinical Evaluation. Asian Journal of Pharmaceutical and Clinical Research 2011; 4:29-33.

60. Santos VR, Pimenta FJ, Aguiar MC, do Carmo MA, Naves MD, Mesquita RA. Oral candidiasis treatment with Brazilian ethanol propolis extract. Phytother Res 2005; 19:652-654.

61. Santos VR, Gomes RT, de Mesquita RAet al. Efficacy of Brazilian propolis gel for the management of denture stomatitis: a pilot study. Phytother Res 2008; 22:1544-1547.

62. Paul IM, Beiler J, McMonagle A, Shaffer ML, Duda L, Berlin CM, Jr. Effect of honey, dextromethorphan, and no treatment on nocturnal cough and sleep quality for coughing children and their parents. Arch Pediatr Adolesc Med 2007; 161:1140-1146.

63. Shadkam MN, Mozaffari-Khosravi H, Mozayan MR. A comparison of the effect of honey, dextromethorphan, and diphenhydramine on nightly cough and sleep quality in children and their parents. J Altern Complement Med 2010; 16:787-793.

64. Crisan I, Zaharia CN, Popovici Fet al. Natural propolis extract NIVCRISOL in the treatment of acute and chronic rhinopharyngitis in children. Romanian journal of virology 1995; 46:115-133.

65. Rajan TV, Tennen H, Lindquist RL, Cohen L, Clive J. Effect of ingestion of honey on symptoms of rhinoconjunctivitis. Ann Allergy Asthma Immunol 2002; 88:198-203.
66. Saarinen K, Jantunen J, Haahtela T. Birch pollen honey for birch pollen allergy--a randomized controlled pilot study. Int Arch Allergy Immunol 2011; 155:160-166.
67. Lazim N, Abdullah B, Salim R. The effect of Tualang honey in enhancing post tonsillectomy healing process. An open labelled prospective clinical trial. Int J Pediatr Otorhinolaryngol 2013; 77:457-461.
68. Ozlugedik S, Genc S, Unal A, Elhan AH, Tezer M, Titiz A. Can postoperative pains following tonsillectomy be relieved by honey? A prospective, randomized, placebo controlled preliminary study. Int J Pediatr Otorhinolaryngol 2006; 70:1929-1934.
69. Robson V, Yorke J, Sen RA, Lowe D, Rogers SN. Randomised controlled feasibility trial on the use of medical grade honey following microvascular free tissue transfer to reduce the incidence of wound infection. Br J Oral Maxillofac Surg 2012; 50:321-327.
70. Marchisio P, Esposito S, Bianchini Set al. Effectiveness of a propolis and zinc solution in preventing acute otitis media in children with a history of recurrent acute otitis media. International journal of immunopathology and pharmacology 2010; 23:567-575.
71. Erejuwa OO, Sulaiman SA, Ab Wahab MS. Honey: a novel antioxidant. Molecules 2012; 17:4400-4423.
72. Strigari L, Pedicini P, D'Andrea Met al. A new model for predicting acute mucosal toxicity in head-and-neck cancer patients undergoing radiotherapy with altered schedules. Int J Radiat Oncol Biol Phys 2012; 83:e697-702.
73. Raber-Durlacher JE, Elad S, Barasch A. Oral mucositis. Oral Oncol 2010; 46:452-456.
74. Worthington HV, Clarkson JE, Eden OB. Interventions for preventing oral mucositis for patients with cancer receiving treatment. Cochrane Database Syst Rev 2007:CD000978.
75. Kwakman PH, de Boer L, Ruyter-Spira CPet al. Medical-grade honey enriched with antimicrobial peptides has enhanced activity against antibiotic-resistant pathogens. Eur J Clin Microbiol Infect Dis 2011; 30:251-257.
76. Kwakman PH, Te Velde AA, de Boer L, Vandenbroucke-Grauls CM, Zaat SA. Two major medicinal honeys have different mechanisms of bactericidal activity. PLoS One 2011; 6:e17709.
77. Cooper RA, Halas E, Molan PC. The efficacy of honey in inhibiting strains of Pseudomonas aeruginosa from infected burns. The Journal of burn care & rehabilitation 2002; 23:366-370.
78. Cooper RA, Jenkins L, Henriques AF, Duggan RS, Burton NF. Absence of bacterial resistance to medical-grade manuka honey. Eur J Clin Microbiol Infect Dis 2010; 29:1237-1241.
79. Maeda Y, Loughrey A, Earle JAet al. Antibacterial activity of honey against community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA). Complementary therapies in clinical practice 2008; 14:77-82.
80. Alandejani T, Marsan J, Ferris W, Slinger R, Chan F. Effectiveness of honey on Staphylococcus aureus and Pseudomonas aeruginosa biofilms. Otolaryngol Head Neck Surg 2009; 141:114-118.

81. Taylor JA, Weber W, Standish Let al. Efficacy and safety of echinacea in treating upper respiratory tract infections in children: a randomized controlled trial. JAMA : the journal of the American Medical Association 2003; 290:2824-2830.
82. Hemila H, Chalker E. Vitamin C for preventing and treating the common cold. Cochrane Database Syst Rev 2013; 1:CD000980.
83. Smith SM, Schroeder K, Fahey T. Over-the-counter (OTC) medications for acute cough in children and adults in ambulatory settings. Cochrane Database Syst Rev 2012; 8:CD001831.
84. Tonks A, Cooper RA, Price AJ, Molan PC, Jones KP. Stimulation of TNF-alpha release in monocytes by honey. Cytokine 2001; 14:240-242.
85. Molan PC. Potential of honey in the treatment of wounds and burns. American journal of clinical dermatology 2001; 2:13-19.
86. Nam JG, Lee TH, Kwon JKet al. Effect of fibrin-coated collagen fleece (TachoComb) on pain and bleeding after adenotonsillectomy in children. Acta Otolaryngol 2011; 131:1293-1298.
87. Segal N, Puterman M, Rotem Eet al. A prospective randomized double-blind trial of fibrin glue for reducing pain and bleeding after tonsillectomy. Int J Pediatr Otorhinolaryngol 2008; 72:469-473.
88. O'Flaherty JE, Lin CX. Does ketamine or magnesium affect posttonsillectomy pain in children? Paediatric anaesthesia 2003; 13:413-421.
89. Hamunen K, Kontinen V. Systematic review on analgesics given for pain following tonsillectomy in children. Pain 2005; 117:40-50.
90. Kelley PE. Painless tonsillectomy. Current opinion in otolaryngology & head and neck surgery 2006; 14:369-374.
91. Singh M, Das RR. Zinc for the common cold. Cochrane Database Syst Rev 2013; 6:CD001364.
92. Gulani A, Sachdev HS. Zinc supplements for preventing otitis media. Cochrane Database Syst Rev 2012; 4:CD006639.
93. Hoarau G, Pelloux I, Gayot Aet al. Two cases of type A infant botulism in Grenoble, France: no honey for infants. Eur J Pediatr 2012; 171:589-591.
94. Abdulla CO, Ayubi A, Zulfiquer F, Santhanam G, Ahmed MA, Deeb J. Infant botulism following honey ingestion. BMJ Case Rep 2012; doi 10.1136/ bcr.11.2011.5153

CHAPTER 3

-

The contribution of α-dicarbonyl compound dependent radical formation to the antiseptic effect of honey

Darius Henatsch, Gertjan J.M. den Hartog, Adriaan M. Duijvestijn, Petra F. Wolffs, Esther Phielix, Robert J. Stokroos, Jacob J. Briedé

Journal of Functional Foods. 2018 Jun;45(6):239-246

Abstract

Honey is known for its wound healing potential. In this study we investigated free radical formation and inhibition in different honeys and the influence on bacterial growth inhibition. α-Oxaldehydes and free amino acids were shown to be present in manuka and non-manuka honeys. With electron spin resonance we measured that methylglyoxal (MG) and glyoxal (GO) form different carbon centered radicals under the influence of either hydrogen peroxide or arginine and lysine. These radicals were also formed in a combination of α-oxaldehydes and amino acids in artificial honey, which was quantified by the cytochrome C assay. Also, a strong bactericidal activity for MG and GO (minimum concentration 0.6 and 1.25 mM) was shown against different bacterial strains. Interestingly, honeys with a high MG content were shown to be better free radical scavengers for hydroxyl and superoxide radicals, implying a complex mechanism for free radical donation and scavenging in honey.

Introduction

A growing challenge in the treatment of infected and chronic wounds is increasing antimicrobial resistance[1]. This expedites the need for novel antiseptic strategies. Honey is probably the oldest wound dressing known by humans[2], with especially strong antiseptic properties, without the formation of bacterial resistance[3]. Additionally, antioxidative and immune-modulatory properties[4] lead to it's wound healing capacity[5,6]. Composition of compounds of different honeys varies strongly, dependent on the source and environmental conditions[4]. Based on their antibacterial properties, medical honeys can be classified into those exerting peroxide and those exerting non-peroxide cytotoxic effects[7], both used and evaluated for their healing capacity in a broad range of acute and chronic wounds, as well as under inflammatory conditions[8-11].

Honeys with peroxide dependent cytotoxic properties have elevated honeybee glucose oxidase activity and produce relatively high amounts of hydrogen peroxide. In these honeys a cytotoxic effect on bacteria was found to be hydroxyl radical dependent, which was enhanced by trace metals and polyphenols[12,13]. Honeys, which are mainly studied for their non-peroxide antibacterial activity are honeys from the nectar of the Manuka *(Leptospermum scoparium)* plant[14]. These honeys contain high amounts of methylglyoxal (MG) due to enhanced levels of dihydroxyacetone, found in Manuka flowers nectar[7]. In Manuka honey, hydrogen peroxide accumulation is low, because of the inhibiting effect of MG on glucose oxidase activity[15]. Other α-dicarbonyls in honeys are glyoxal (GO) and 3-deoxyglucosone (3-DG)[16], which can be generated during glucose degradation[17,18]. These molecules are more reactive than their parent sugars and are responsible for the generation of free radicals by non-enzymatic reactions[19], by interacting with amino acid groups of proteins. This induces the formation of advanced glycation end products[20]. The reaction of MG with amino acids (AA) as lysine (Lys)[21] or alanine[22] can lead to cross-linked radicals, MG anion radicals and superoxide radicals under aerobic conditions. The formation of these reactive oxygen species (ROS) induces DNA damage[23] and is supposed to have an antibacterial effect, via the alteration of the structure of bacterial fimbriae and flagella[24].

Little is known about the interaction of MG and other α-oxoaldehydes in honey with sugars and AA. In our hypothesis, the high accumulation of carbonyl compounds leads to “carbonyl stress”, which could be the main bactericidal factor in medical honey. Therefor this study addresses 3 different research questions, regarding ROS formation induced by honeys with different dicarbonyl compositions, their antiseptic effects and antioxidative potential. The first aim is to examine the formation of different radicals in artificial honey, induced by the interaction of MG, GO and 3-DG with the amino acids Lys and arginine (Arg). The second aim is to determine α-oxoaldehyde and free amino acid content of medical manuka and non-manuka honeys and measure the inhibitory capacity of these honeys and oxaldehyde solutions on different bacteria. The third aim is to identify the free radical quenching potential of peroxide and non-peroxide honeys associated with the AA and oxaldehyde content.

Materials and methods

Honey samples and artificial honey

Four different honeys were selected: (1) Manuka honey 30+ (M30) and (2) 550+ (M550) (MGO Manuka Honey, Manuka Health New Zealand), (3) Revamil honey (RH) (Bfactory Health Products B.V, Rhenen, The Netherlands) and (4) supermarket (flower) honey (FH) (Plus supermarket, The Netherlands). Honeys were chosen to compare medical honeys (Manuka honeys with a high and low MG concentration to a non-Manuka medical honey (RH)) to a non-medical honey (FH). Previously it was shown that RH is a peroxide honey, which produces up to 5 mM hydrogen peroxide upon dilution after 24h[25], whereas non-peroxide Manuka honeys did not produce any hydrogen peroxide[26]. In addition, an artificial honey (AH) was prepared as a control sugar solution. This solution (100g) was prepared by dissolving 1.5 g sucrose, 7.5 g maltose, 40.5 g D-fructose and 33.5 g D-glucose (Sigma-Aldrich, Germany), in 17 mL of sterile, deionized water, which is in line with the proportions of the 4 major sugars in natural honeys and used by other authors[27,28].

Measurement of α-oxoaldehydes

Different α-oxoaldehydes were measured as described[29]. In short, honey solutions (10% w/v) were deproteinized with perchloric acid (Sigma-Aldrich, Germany), and derivatized with o-phenylenediamine (Merck, Germany). Derivatized MG, along with GO and 3-DG were analyzed by ultra performance liquid chromatography (Acquity Ultra Performance LC from Waters) and detected using Tandem mass spectrometry (Xevo TQ MS from Waters). Quantification of the α-oxoaldehydes was performed by calculating the peak area ratio of each unlabeled peak area to the corresponding internal standard peak area.

Measurement of free amino acids in honey

The amino acid concentrations of different honeys were determined with a fully-automated high-performance liquid chromatography (HPLC) and pre-column o-phthaldialdehyde derivatization. A 150 mm x 4.6 mm I.D. HPLC column was used filled with 2-3 μm Spherisorb ODS II packing material. Methodology applied as previously reported[30]. Honeys were diluted in methanol (2:1).

Assessment of hydroxyl and superoxide radical scavenging by honey

The antioxidant activity (radical quenching) of different concentrations of honeys was determined using electron spin resonance (ESR) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap. DMPO was purified by a charcoal treatment. In order to produce a hydroxyl radical, the Fenton reaction was mimicked by reacting 100 μM H_2O_2 and 100 μM $FeSO_4$. Superoxide radicals were produced by reacting 3.3 μM PMS and 50 μM NADH. After the addition of honeys and DMPO (100 mM), the reaction mixture was transferred to a sealed glass capillary (100 μl, Brand AG Wertheim, Germany) and measured immediately at room temperature on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity cavity and 12 kW power supply operating at X band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were: magnetic field: 3490 G; scan range: 60 G; modulation amplitude: 1 G; receiver gain: 1 x 105; microwave frequency: 9.85 GHz; power: 50 mW; time constant: 40.96 ms; scan time: 20.97 s; number of scans: 20. Radical signals in the spectra were quantified (in arbitrary units) through peak surface measurements using the WIN-EPR spectrum manipulation program.

Assessment of α-oxaldehyde dependent radical formation

In order to determine radical formation of α-oxoaldehydes and AA, different concentrations of MG (6.9 mM or 200 mM), GO (200 mM or 6.9 M) and 3-DG (3.82 mM) were mixed with Lys (200 mM) and Arg (100 mM) and AH (30% w/v). Where indicated, 100 μM of H_2O_2 was added. Subsequently, 100 mM of DMPO was added and the reaction mixture was transferred to a sealed glass capillary and measured immediately using ESR as described.

Superoxide radical production: Cytochrome C assay

Superoxide formation was quantified by monitoring the rate of cytochrome c reduction using a Varian Cary 20 UV VIS spectrophotometer in incubations containing MG (200 mM) or honey (25% w/v) with or without AA (Arg 100 mM and Lys 200 mM). Non superoxide dependent (direct) reduction of cytochrome c by MG or artificial honey was corrected for by determining the rate of cytochrome c reduction in the presence of superoxide dismutase 1 (10 U/ml).

Broth micro-dilution assay

Bactericidal activity of honeys and different solutions were assessed against different bacteria in a 2-fold microbroth dilution assay. The following strains were tested: *Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeroginosa), Staphylococcus aureus (S. aureus)*. Honeys were diluted in sterile demi water to a concentration of 50% (w/v). All α-oxoaldehydes and AA were diluted in sterile PBS (Gibco, 1x, pH 7.2). Of all solutions 2-fold serial dilutions (50 μl) in physiological salt were made in 96-well plates. Overnight cultures of all strains were diluted to 1.5×10^5 CFU/ml in double concentrated Mueller Hinton II Broth (Cation-Adjusted) (BD) and 50 μl per well was added to the serial dilutions (one strain per serial dilution). The 96-well plates were incubated overnight at 37° C. The minimal inhibitory dilution (MID) was determined as the first dilution at which no growth was visible. All micro-dilution assays were performed *in duplo*.

Statistics

All data are presented as mean ± SD. All experiments were carried out in 3-fold, if not mentioned otherwise. Data fitting was performed with GraphPad prism 5. Significance was tested via 2-way ANOVA with post-hoc Hochberg correction and considered significant with $p < 0.05$. Correlation coefficients were determined using GraphPad prism 5 to estimate the correlation between variables.

Results

Interactions between dicarbonyl compounds, amino acids and ROS

Our hypothesis was to study the interaction between AA and α-oxoaldehydes in a honey environment and its contribution to the antiseptic potential of honey. The 3 dicarbonyl compounds MG, GO and 3-DG are more reactive, compared to their parent sugars and may form cross-linked products with amino acid groups of proteins. In addition, MG is also known to form ROS by reacting with AA[23]. We used ESR, with DMPO as spin trap to quantify and identify the formation of radicals by the interaction of dicarbonyl compounds with AA either in the presence of hydrogen peroxide or in an AH environment. In the presence of H_2O_2, MG showed a clear spectrum consisting of 6 different peaks (Fig. 1A), resembling a typical DMPO spin-trapped carbon-centered radical. This was supported by the hyperfine coupling constants, *a*N = 15.0 ± 0.2G and *a*H = 18.5 ± 0.2G, comparable to what was detected before[19,31] and most probably will be DMPO-C(O)-C(O)-CH_3 radical[31]. The radicals formed by the reaction of MG and Lys (Fig. 1B) without the addition of H_2O_2 showed a multicomponent hyperfine structure that exists from a mixture of DMPO-C(O)-C(O)-CH_3 and the Schiff base cation radical MG dialkylimine obtained from the reaction between MG and Lys[21]. In contrast, when MG reacted with Arg, a spectrum (Fig. 1C) was obtained that showed a mixture of most probably again the DMPO-C(O)-C(O)-CH_3 radical (*a*N = 15.0 ± 0.3G and *a*H = 18.6 ± 0.2G) and hydroxyl radicals (*a*N = 14.6 ± 0.3G and *a*H = 14.7 ± 0.4G). When GO reacted with H_2O_2 a much lower radical signal (Fig. 1D) is obtained compared to MG. Again a carbon-centered radical was detected with slightly different hyperfine coupling constants, *a*N = 15,4 ± 0,2G and *a*H = 20,7 ± 0,4G if compared to MG, most likely this is the DMPO-C(O)-C(O)-CH radical. Since the signal is much lower, reactivity of GO with H_2O_2 seems to be much lower. GO in combination with Lys showed a clear 6 peak spectrum (Fig. 1E), with hyperfine coupling constants of *a*N = 15.9 ± 0.3G and *a*H = 23.5 ± 1.0G. Taken into account the broadness of these peaks, they might reflect different overlapping signals of carbon-centered radicals. The combination with Arg and GO (Fig. 1F), just like MG, showed a spectrum consisting of a mixture of a broad carbon-centered radical (*a*N = 15.9 ± 0.3G and *a*H = 23.5 ± 1.0G) and hydroxyl radicals (*a*N = 14.6 ± 0.3G and *a*H = 14.7 ± 0.4G). 3-DG in the presence of H_2O_2 or AA did not show DMPO trapped radicals, indicating a very low reactivity of 3-DG with H_2O_2 and Lys and Arg. So overall this indicates that regarding the reactivity with H_2O_2, MG has a higher reactivity compared to GO and 3-DG. In combination with the amino acid Lys, reactivity of GO seems to be increased. This hydroxyl radical lowering effect was confirmed in ESR experiments were we analyzed radical formation in the hydroxyl generating system and the combination of MG with GO, 3-DG, Arg and Lys (Fig. 1G) or in the combination MG, GO, 3-DG, Arg, Lys with 30% AH (Fig. 1H).

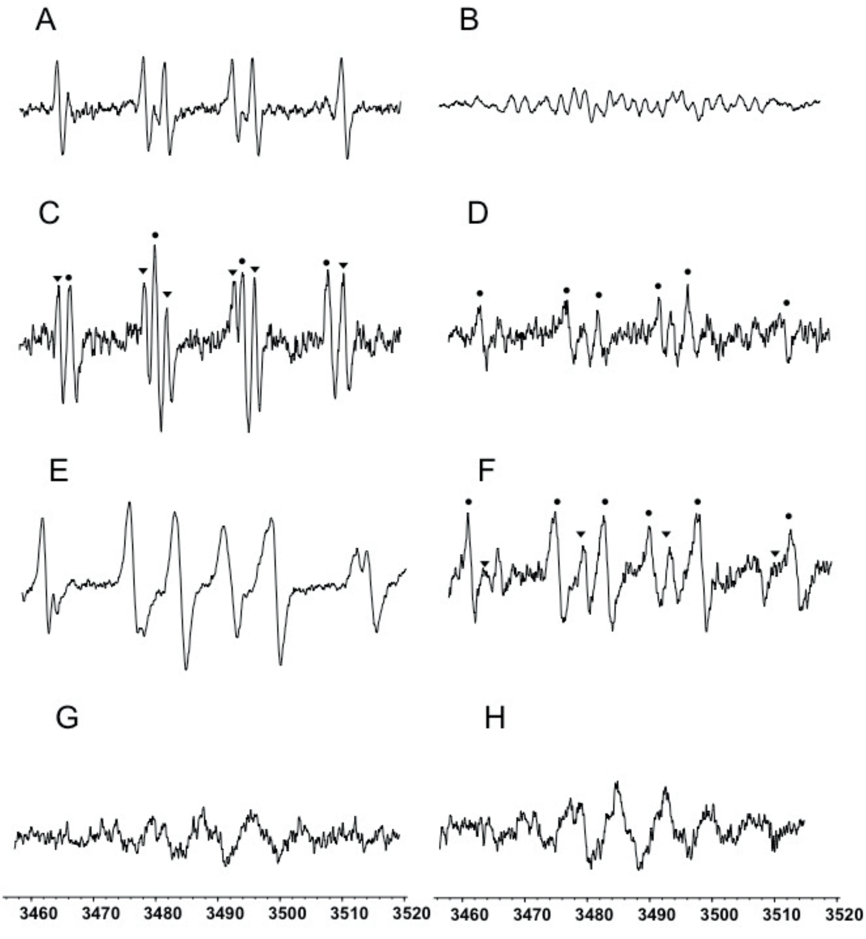


Fig. 1: ESR spectra for A) MG (6.9 mM) and H_2O_2 (100 μM), hyperfine coupling constants: *a*N=15.0 ± 0.2G and *a*H=18.5 ± 0.2G (carbon-centered radical) B) MG (200 mM) and Lys (200 mM) C) MG (200 mM) and Arg (100 mM), hyperfine coupling constants *a*N=15.0 ± 0.3G and *a*H=18.6 ± 0.2G (carbon-centered radical ●) and *a*N=14.6 ± 0.3G and *a*H=14.7 ± 0.4G (hydroxyl radical ▼) D) GO (6.9 M) and H_2O_2 (100 μM), hyperfine coupling constants: *a*N=15.4 ± 0.2G and *a*H=20.7 ± 0.4G (carbon-centered radical ●) E) GO (200 mM) and Lys (200 mM), hyperfine coupling constants: *a*N=15.9 ± 0.3G and *a*H=23.5 ± 1,0G (carbon-centered radical) F) GO (200 mM) and Arg (100 mM), hyperfine coupling constants: *a*N=15.9 ± 0.3G and aH=23.5 ± 1.0G (carbon-centered radical ●) and *a*N=14.6 ± 0.3G and *a*H=14.7 ± 0.4 (hydroxyl radical ▼) G) MG (200 mM), GO (200 mM), 3-DG (3.82mM), Arg (100 mM) and Lys (200 mM) H) MG, GO, 3-DG, Arg, Lys (concentrations as in G) with 30% AH.

After determining the interactions with H_2O_2 we studied how the combinations of dicarbonyl compound in different combinations in an AH environment can contribute to the production of superoxide radicals, which was measured by the cytochrome C assay. We detected that mainly the combination of MG and GO with a combination of Lys/Arg led to superoxide radical production (Fig. 2). Interestingly, the highest superoxide production was measured in a mixture of all different α-oxaldehydes with AA in a context of AH (25% w/v). A possible reaction of MG and AA that contribute to Schiff base and superoxide production is shown in Fig. 3.

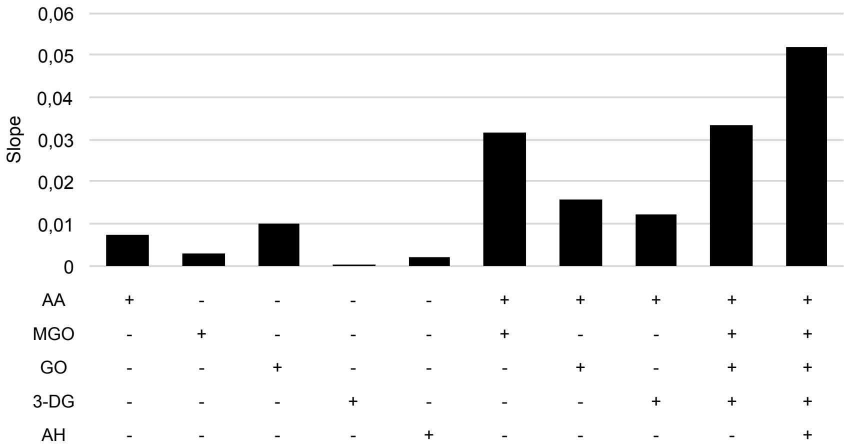


Fig. 2: Rate of superoxide induced cytochrome c reduction (dA560/dt) in incubations with different combinations of dicarbonyl compounds with or without AA (mixture of Lys and Arg).

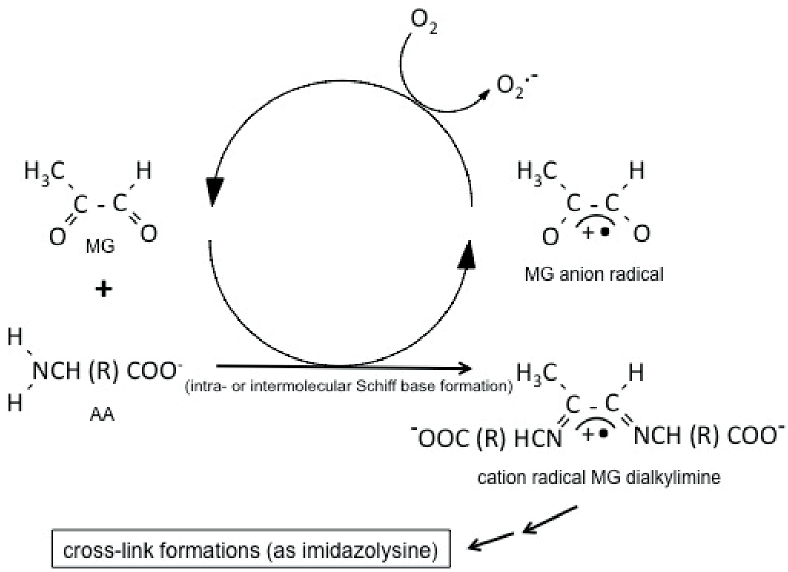


Fig. 3. Possible reaction of MG and amino acids, underlying Schiff base and superoxide radical formation.

Non-peroxide antiseptic effect of honey

We chose peroxide and non-peroxide medical honeys, due to their differences in radical formation. The antibacterial potential of RH is dependent on it's hydrogen peroxide activity, but also low MG content, pH and other antimicrobial compounds[25]. Manuka honey deploys it's antiseptic capacity mainly by MG, but also other unidentified compounds[26]. In Manuka honey the non-peroxide antibacterial activity is directly correlated to it's MG content[32]. To our knowledge there is no information about the interaction of GO and 3-DG in the non-peroxide honey context. We determined the levels of all 3 α-oxaldehydes MG, GO and 3-DG of all 4 honeys. As expected, the highest concentration of MG was found in M550 (497 mg/kg), followed by M30 (132 mg/kg), as shown in Table 1.

	α-oxaldehydes		
Honeys	MG (mg/kg)	GO (mg/kg)	3-DG (mg/kg)
M550	497.1 (4.1)	14.5 (0.6)	568.4 (3.8)
M30	132.2 (0.2)	14.4 (0.2)	618.0 (6.2)
RH	29.3 (0.1)	27.3 (0.1)	221.6 (11.9)
FH	38.4 (2.0)	15.9 (0.7)	687.3 (4.3)

Table 1: α -oxaldehydes in honey (SD shown in brackets).

3

The medical and non-medical non-manuka honeys had much lower quantities of MG (29 and 38 mg/kg respectively). Interestingly, GO content was found to be similar and low in all 4 honeys, at concentrations ranging from 14-27 mg/kg, whereas, concentrations of 3-DG were high in M550, M30 and FH (around 600 mg/kg) and lower in RH (222 mg/kg). The concentration of MG in these honeys did not correlate to concentrations of GO or 3-DG (R^2=0.2, p=0.5 / R^2=0.05, p=0.8 respectively). In addition, we determined the free amino acid concentration in all honeys. We measured variable concentrations in different honeys (Table 2). In all honeys, higher concentration were measured for GLU, NVAL and PHE (ranging from 3.2-58.3 mmol/100g) and low concentrations for aAB, MET and TRP (ranging from 0.0-0.81 mmol/100g). The total free amino acid content was 101 (M550), 101 (M30), 35 (RH) and 126 mmol/100g (FH), showing a lower concentration of free AA in the medical non-manuka honey. We were especially interested in the concentrations of Arg and Lys, due to their possible role in ROS production in the presence of MG[23]. Arg concentrations were ranging from 0.4-8.3 mmol/100g and Lys concentrations from 1.7-3.4 mmol/100g. As the total free amino acid content, Arg and Lys concentrations were both highest in M550 and lowest in RH.

Honey	Free amino acids (mmol/100g)																				
	GLU	ASN	SER	GLN	HIS	GLY	THR	CIT	ARG	ALA	TAU	aAB	TYR	VAL	MET	NVAL	ILE	PHE	TRP	LEU	ORN	LYS
M550	10,81	3,77	5,00	4,77	4,50	2,53	1,06	2,58	8,32	4,63	0,81	0,20	4,32	4,14	0,47	16,64	2,08	17,22	0,00	1,61	1,86	3,41
M30	15,94	3,28	5,15	4,70	0,43	3,31	1,49	2,79	0,79	5,81	1,22	0,37	4,44	3,21	0,81	19,61	1,59	19,60	0,71	1,72	1,83	2,12
RH	3,23	0,00	2,05	0,00	0,00	1,22	1,06	0,19	0,42	1,07	0,39	0,00	0,84	0,59	0,22	18,40	0,28	2,76	0,00	0,57	0,28	1,68
FH	8,51	5,01	5,09	2,62	0,28	2,48	2,81	0,04	0,86	6,24	3,16	0,36	6,43	3,80	0,56	11,38	2,48	58,31	0,00	3,13	0,28	2,17

Table 2: Amino acid content of different honeys, determined with a fully-automated HPLC.

To test our hypothesis that the non-peroxide dependent antiseptic effect of honeys is mainly caused by α-oxoaldehyde induced ROS, we tested the inhibitory activity of all different honeys and different α-oxoaldehyde solutions on the bacteria *E. coli*, *S. aureus* and *P. aeroginosa*. All medical honeys showed inhibitory activity on all pathogens at concentrations ranging from 3.1-12.5%. M550 had highest effect with a MID of 3.1%. FH was the only honey, not inhibiting the growth of all species at concentrations of 25%. In order to test the inhibitory effect of α-oxoaldehydes and AA, we tested solely all α-oxoaldehydes as well as in combination with AA. In addition we chose a concentration mixture, which was similar to concentrations of α-oxoaldehydes and AA in manuka honey. MG and GO both showed high inhibitory activity, and inhibited bacteria at concentrations ranging from 0.6 to 2.5 mM and 1.25-5 mM respectively (Table 3). Because of a maximum stock concentration of 4 mM, the highest possible concentration to test for 3-DG was 2 mM, where we found no inhibitory activity. Lys/Arg at concentrations of 10 mM neither inhibited bacterial growth. The MID for AA were ranging from 12.5-25 mM (data not shown). Contrary to our expectation from ESR and cytochrome C experiments, where we saw strong (superoxide) radical formation by the addition of AA to GO or MG, this did not enhance but reduce their inhibitory activity on bacterial growth. The combination of all 3 α-oxoaldehyde, in an artificial and a honey-like mixture showed MID of 0.6 mM.

	Minimal inhibitory dilutions		
Mixtures (highest conc.)	E. coli	S. aureus	P. aeroginosa
PBS	R	R	R
Arg/Lys (10/10 mM)	R	R	R
MGO (10 mM)	1/16	1/16	1/4
GO (10 mM)	1/8	1/8	1/2
3-DG (2 mM)	R	R	R
MGO/Lys/Arg (10/10/10 mM)	R	R	R
MGO/Lys/Arg (10/5/5 mM)	R	R	R
GO/Lys/Arg (10/10/10 mM)	R	R	R
GO/Lys/Arg (10/5/5 mM)	R	R	R
3-DG/Lys/Arg (2/10/10 mM)	R	R	R
3-DG/Lys/Arg (2/1/1 mM)	R	R	R
MGO/GO/3-DG/Lys/Arg (10/10/2/10/10 mM)	1/4	1/4	1/2
MGO/GO/3-DG (10/10/2 mM)	1/32	1/32	1/8
MGO/GO/3-DG/Lys/Arg (2/0.1/1/0.05/0.05 mM)	1/4	1/4	R
MGO/GO/3-DG (2/0.1/1 mM)	1/4	1/4	R
M550 (25%)	1/4	1/8	1/2
M30 (25%)	1/2	1/4	1/4
RH (25%)	1/2	1/2	1/2
FH (25%)	1/2	1/2	R
PBS phosphate buffered saline, R resistant			

Table 3: Minimal inhibitory dilutions of honeys and different oxaldehyde solutions. Duplicate measurements were identical.

Oxidative stress and radical quenching capacity of honey: the double-edged sword

As mentioned earlier, in a wound environment honey seems to deploy it's inhibitory potential on pathogens mainly by hydrogen peroxide and by α-oxoaldehyde dependent ROS development[25,26.] The wound healing potential of honey is based on it's antibacterial but also antioxidative capacity.

This capacity is based on sugars, phenolic compounds, AA[33] and other components[28,34]. We showed that oxaldehydes in combination with and without hydrogen peroxide and AA form different radicals, which contribute to the antiseptic effect of honeys. Whereas it is known that in chronic wounds not only an antiseptic therapy is important for the enhancement of healing, but also the addition of a free radical scavenging substance[35]. We used ESR to show the radical quenching capacity of both, manuka and non-manuka honeys on hydroxyl and superoxide radicals.

Hydroxyl radicals were generated by the Fenton reaction (Fe^{2+} / H_2O_2) and trapped with the spin-trap DMPO. For hydroxyl radicals, the typical 1:2:2:1 spectrum of the ESR adduct $DMPO^{\cdot}$-OH was seen. All honey samples were dissolved with pure water to the final concentrations of 25%, 10%, 5% and 1% (w/v) and added to radical producing agents. The dose dependent quenching of hydroxyl radicals is shown in Fig. 4a. Superoxide radicals were produced by a non-enzymatic PMS/NADH system. After trapping with DMPO, initially a $DMPO^{\cdot}$-OOH signal was detected, which was subsequently converted into a $DMPO^{\cdot}$-OH signal[36]. The dose dependent quenching of superoxide radicals is shown in Fig. 4b.

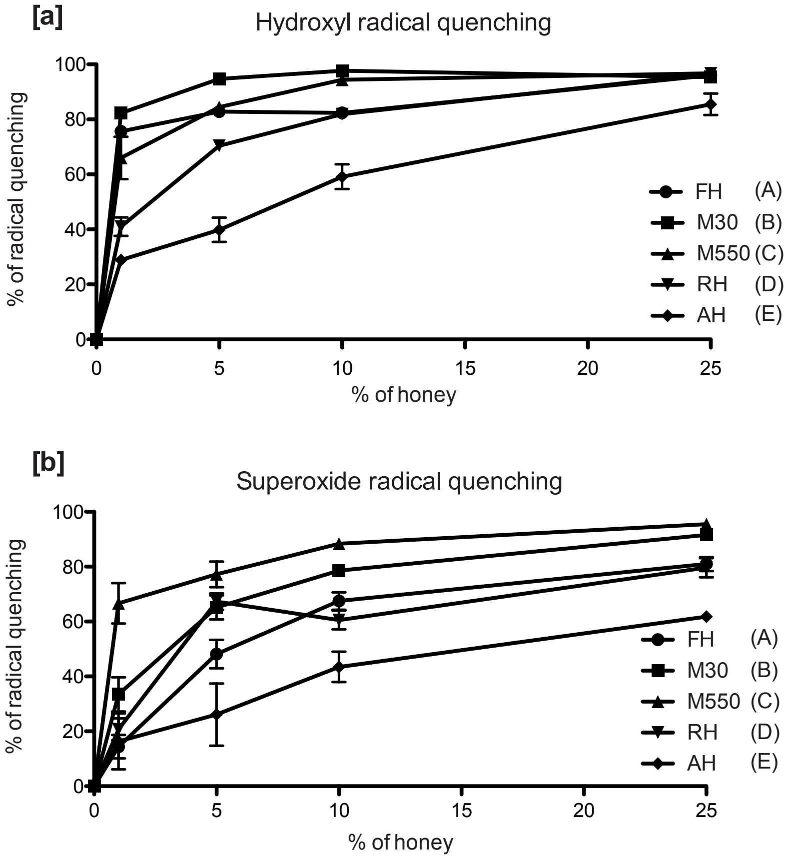


Fig. 4: Radical quenching of (a) hydroxyl and (b) superoxide radicals of different concentration of peroxide and non-peroxide honeys. Radical formation and quenching was measured using ESR and DMPO as a spin trap. Statistical differences for 5% honey concentration were calculated with ANOVA with post-hoc Hochberg correction: (a) [AB, AE, BC, BD, BE, CD, CE < 0.0001; AD, DE < 0.001; AC = NS]; (b) [AC, AD, BE, CE, DE < 0.05; AB, AE, BC, BD, CD = NS].

It was shown that both manuka honeys (M30, M550) had the highest capacity of radical quenching for both hydroxyl and superoxide radicals. The lowest quenching potential was seen in AH. The overall quenching of superoxide radicals was lower, compared to hydroxyl radicals. High differences in quenching potential are only seen in low concentrations of honey. At concentrations of > 25%, all honeys are good radical scavengers. The maximum difference of hydroxyl radical and superoxide quenching capacity was seen at 5% of honey concentration. Statistical outcomes are presented in figure legend of Fig. 4.

The reduction of hydroxyl and superoxide radicals by rising concentrations of honeys was further accompanied by the increase of a DMPO.-CH3 signal. At the percentage of 5% honey's quenching potential of hydroxyl radicals, at which the maximum difference of quenching potential between honey was seen, was not correlated to MG content ($R2 = 0.08$, $p = 0.72$), total oxaldehyde content ($R2 = 0.45$, $p = 0.33$), or total AA content ($R2 = 0.51$, $p = 0.28$). The same quenching potential of superoxide radicals was also not correlated to MG, total oxaldehyde, or total AA content.

Discussion

In this study we showed that of the 3 α-oxaldehydes MG, GO and 3-DG, mainly MG and GO form different radicals in reaction with Lys and Arg in a honey-like environment, which leads to the production of ROS. All tested peroxide and non-peroxide honeys show similar inhibitory effects on different strains of bacteria, in which both MG and GO were shown to be strong antibacterial agents. In addition non-peroxide honeys turn out to be good free radical scavengers, even at low concentration.

Honey is a promising antiseptic agent[3], with additional wound healing capacities[5,6] and was 'rediscovered' as a wound healing agent[37]. In the non-peroxide Manuka honey, the α-oxaldehyde MG is highly abundant[7], and has gained special interest, due to it's antibacterial capacity[32]. It is important to distinguish between peroxide and non-peroxide honey, because of different radical producing properties. In peroxide honeys, which are low in MG, dilution leads to an enhanced action of glucose oxidase, which results in the conversion of D-glucose, water and oxygen to D-gluconic acid and hydrogen peroxide[38], which is highest at 30% diluted honey[38]. Hydrogen peroxide plays a role in wound healing during inflammation, angiogenesis and re-epithelialization,[39] and generates ROS by the Fenton reaction. ROS is also produced by cells of the innate immune system as a defense mechanism against bacteria[40], the same mechanism, which is present in honey.

In non-peroxide honeys, ROS are produced by another, oxaldehyde dependent mechanism. Reactive α-dicarbonyl compounds are known from sugar-amino acid model systems[41], but can also be found in varying concentrations in different sorts of honeys[42,43]. Their precursors are Amadori compounds and reducing sugars[16].

We showed that all 3 α-oxaldehydes alone do not form any radicals, but under the influence of H_2O_2, both MG and GO form slightly different carbon-centered

radicals. Also MG was more reactive if compared to GO. In the combination with Lys and Arg, both MG and GO formed different radicals, without the influence of H_2O_2. In the reactions of GO with AA, carbon-centered and hydroxyl radicals evolve, whereas MG seems to forms the Schiff base cation radical MG dialkylimine. This is very important, because the formed free radical intermediate MG anion radical could reduce oxygen further to superoxide radicals. It seems that the Schiff bases formation is an essential step for this cross-linking process[22]. In this process, it is also possible that superoxide is formed by the reaction of oxygen and dialkylimine cation radicals[21]. We further proved that radical formation of α-oxaldehydes in combination with AA happens in an environment of different sugars (artificial honey). Different solutions of oxaldehydes with AA also lead to the net production of superoxide radicals, and we showed that this production was highest in an artificial honey environment. This could imply that this is the most important mechanism for ROS production in medical honeys. Oxaldehydes and amino acids were processed in media with a pH ranging from 7.0 to 7.4. It was shown that superoxide is formed by the reaction of MG and amino acids under high pH, but also physiologic pH conditions[21]. In addition, a good reactivity was shown for MG and GO with H_2O_2 with a pH of 7.4[31]. In wound conditions, where honey is added to wound fluids, an even more acid condition can be expected. In line with other studies[28,33], we showed that honeys contain different quantities of free AA and that superoxide radicals are formed by oxaldehydes in the presences of Lys or Arg[44].
We tested the minimal inhibitory activity of different honeys and α-oxaldehyde and AA solutions. M550 honey had the highest and FH the lowest inhibitory capacity on bacterial growth, with MID ranging from 3.1-12.5%. This is in line with other studies, reporting similar MIDs[45,46]. MG and GO, both inhibited bacterial growth at very low concentrations, ranging from 0.6 to 2.5 mM and 1.25-5 mM, respectively. Whereas, against expectations, incubation of bacteria with a combination of different oxaldehydes and AA did not lead to an enhanced antibacterial effect, compared to single oxaldehydes alone. It was shown that reactions between oxaldehydes and amines lead to the production of ROS[47] and that DNA strand breakage may be induced by superoxide anions, produced by a MG-amino acid-Fe^{3+} system[23]. It is possible that the early interaction and binding of MG or GO to AA inhibits further interaction with proteins and trace metals with an influence on modification and fragmentation of proteins[20]. There is little information about bacterial cell permeability and cell membrane interaction with cross-link formations as show in Fig. 3. But it is possible, that extra-cellular radical and Schiff base formation is less bactericidal, compared to intracellular. This could explain our results. We did

however not test the interaction of α-oxaldehydes with proteins, which are present in honey as well[48], to study these Maillard reaction products. In addition we show for the first time that besides MG, also GO could be an important agent in honey and responsible for radical production and bacterial inhibition and not correlated to MG content. This shows that besides the endogenous toxicity of GO under influence of H_2O_2 and oxidative stress[49], GO from honey can play an important role in wound healing as well.

We tested the radical quenching capacity, the trapping of free radicals, of different peroxide and non-peroxide honeys. Both Manuka honeys qualified as very good radical scavengers for both hydroxyl and superoxide radicals. We could not show a correlation to free AA, as reported elsewhere[33] and also not to α-oxaldehydes content.

In conclusion, we showed that the α-oxoaldehydes MG, GO and 3-DG are present in varying concentrations in peroxide and non-peroxide honeys. Mainly MG and GO are responsible for ROS formation, which is dependent on interactions with hydrogen peroxide or AA. These radicals are also formed in an artificial honey environment and are responsible for bacterial growth inhibition. Non-peroxide Manuka honeys also have the highest free radical quenching potential for hydroxyl and superoxide radicals. Overall the wound healing potential of honey is supported by the balance between reactive oxygen radicals scavenging and formation, where α-oxaldehyde/amino acid interactions play an important role. This has to be taken into account for the selection of medical honey for clinical applications.

References

1. Roberts CD, Leaper DJ, Assadian O. The Role of Topical Antiseptic Agents Within Antimicrobial Stewardship Strategies for Prevention and Treatment of Surgical Site and Chronic Open Wound Infection. Adv Wound Care (New Rochelle) 2017; 6:63-71.
2. Molan PC. Re-introducing honey in the management of wounds and ulcers - theory and practice. Ostomy Wound Manage 2002; 48:28-40.
3. Mandal MD, Mandal S. Honey: its medicinal property and antibacterial activity. Asian Pac J Trop Biomed 2011; 1:154-160.
4. Viuda-Martos M, Ruiz-Navajas Y, Fernandez-Lopez J, Perez-Alvarez JA. Functional properties of honey, propolis, and royal jelly. J Food Sci 2008; 73:R117-124.
5. Molan PC. The evidence supporting the use of honey as a wound dressing. Int J Low Extrem Wounds 2006; 5:40-54.
6. Jull AB, Rodgers A, Walker N. Honey as a topical treatment for wounds. Cochrane Database Syst Rev 2008:CD005083.
7. Grainger MN, Manley-Harris M, Lane JR, Field RJ. Kinetics of conversion of dihydroxyacetone to methylglyoxal in New Zealand manuka honey: Part I--Honey systems. Food Chem 2016; 202:484-491.
8. Henatsch D, Wesseling F, Briede JJ, Stokroos RJ. Treatment of chronically infected open mastoid cavities with medical honey: a randomized controlled trial. Otol Neurotol 2015; 36:782-787.
9. Henatsch D, Wesseling F, Kross KW, Stokroos RJ. Honey and beehive products in otorhinolaryngology: a narrative review. Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery 2016; 41:519-531.
10. Jull AB, Cullum N, Dumville JC, Westby MJ, Deshpande S, Walker N. Honey as a topical treatment for wounds. Cochrane Database Syst Rev 2015:CD005083.
11. Malhotra R, Ziahosseini K, Poitelea C, Litwin A, Sagili S. Effect of Manuka Honey on Eyelid Wound Healing: A Randomized Controlled Trial. Ophthal Plast Reconstr Surg 2016.
12. Brudzynski K, Lannigan R. Mechanism of Honey Bacteriostatic Action Against MRSA and VRE Involves Hydroxyl Radicals Generated from Honey's Hydrogen Peroxide. Front Microbiol 2012; 3:36.
13. Brudzynski K, Abubaker K, Miotto D. Unraveling a mechanism of honey antibacterial action: polyphenol/H(2)O(2)-induced oxidative effect on bacterial cell growth and on DNA degradation. Food Chem 2012; 133:329-336.
14. Kwakman PH, Zaat SA. Antibacterial components of honey. IUBMB Life 2012; 64:48-55.
15. Majtan J, Bohova J, Prochazka E, Klaudiny J. Methylglyoxal may affect hydrogen peroxide accumulation in manuka honey through the inhibition of glucose oxidase. J Med Food 2014; 17:290-293.
16. Marceau E, Yaylayan VA. Profiling of alpha-dicarbonyl content of commercial honeys from different botanical origins: identification of 3,4-dideoxyglucoson-3-

ene (3,4-DGE) and related compounds. J Agric Food Chem 2009; 57:10837-10844.
17. Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW. Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. Biochemistry 1995; 34:3702-3709.
18. Thornalley PJ, Langborg A, Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J 1999; 344 Pt 1:109-116.
19. Nakayama M, Saito K, Sato Eet al. Radical generation by the non-enzymatic reaction of methylglyoxal and hydrogen peroxide. Redox Rep 2007; 12:125-133.
20. Lee C, Yim MB, Chock PB, Yim HS, Kang SO. Oxidation-reduction properties of methylglyoxal-modified protein in relation to free radical generation. J Biol Chem 1998; 273:25272-25278.
21. Shumaev KB, Gubkina SA, Kumskova EM, Shepelkova GS, Ruuge EK, Lankin VZ. Superoxide formation as a result of interaction of L-lysine with dicarbonyl compounds and its possible mechanism. Biochemistry (Mosc) 2009; 74:461-466.
22. Yim HS, Kang SO, Hah YC, Chock PB, Yim MB. Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross-linked free radicals. J Biol Chem 1995; 270:28228-28233.
23. Kang JH. Oxidative damage of DNA by the reaction of amino acid with methylglyoxal in the presence of Fe(III). Int J Biol Macromol 2003; 33:43-48.
24. Rabie E, Serem JC, Oberholzer HM, Gaspar AR, Bester MJ. How methylglyoxal kills bacteria: An ultrastructural study. Ultrastruct Pathol 2016; 40:107-111.
25. Kwakman PH, te Velde AA, de Boer L, Speijer D, Vandenbroucke-Grauls CM, Zaat SA. How honey kills bacteria. Faseb J 2010; 24:2576-2582.
26. Kwakman PH, Te Velde AA, de Boer L, Vandenbroucke-Grauls CM, Zaat SA. Two major medicinal honeys have different mechanisms of bactericidal activity. PLoS One 2011; 6:e17709.
27. Cooper RA, Molan PC, Harding KG. The sensitivity to honey of Gram-positive cocci of clinical significance isolated from wounds. Journal of applied microbiology 2002; 93:857-863.
28. Alvarez-Suarez JM, Gonzalez-Paramas AM, Santos-Buelga C, Battino M. Antioxidant characterization of native monofloral Cuban honeys. J Agric Food Chem 2010; 58:9817-9824.
29. Scheijen JL, Schalkwijk CG. Quantification of glyoxal, methylglyoxal and 3-deoxyglucosone in blood and plasma by ultra performance liquid chromatography tandem mass spectrometry: evaluation of blood specimen. Clin Chem Lab Med 2014; 52:85-91.
30. van Eijk HM, Rooyakkers DR, Deutz NE. Rapid routine determination of amino acids in plasma by high-performance liquid chromatography with a 2-3 microns Spherisorb ODS II column. J Chromatogr 1993; 620:143-148.
31. Matsumura Y, Iwasawa A, Kobayashi T, Kamachi T, Ozawa T, Kohno M. The reactivity of alpha-oxoaldehyde with reactive oxygen species in diabetes complications. J Clin

Biochem Nutr 2013; 52:128-132.
32. Cokcetin NN, Pappalardo M, Campbell LTet al. The Antibacterial Activity of Australian Leptospermum Honey Correlates with Methylglyoxal Levels. PLoS One 2016; 11:e0167780.
33. Perez RA, Iglesias MT, Pueyo E, Gonzalez M, de Lorenzo C. Amino acid composition and antioxidant capacity of Spanish honeys. J Agric Food Chem 2007; 55:360-365.
34. Gheldof N, Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. J Agric Food Chem 2002; 50:3050-3055.
35. Salim AS. The role of oxygen-derived free radicals in the management of venous (varicose) ulceration: a new approach. World J Surg 1991; 15:264-269.
36. Ueno I, Kohno M, Mitsuta K, Mizuta Y, Kanegasaki S. Reevaluation of the spin-trapped adduct formed from 5,5-dimethyl-1-pyrroline-1-oxide during the respiratory burst in neutrophils. J Biochem 1989; 105:905-910.
37. Khan FR, Ul Abadin Z, Rauf N. Honey: nutritional and medicinal value. Int J Clin Pract 2007; 61:1705-1707.
38. Bang LM, Buntting C, Molan P. The effect of dilution on the rate of hydrogen peroxide production in honey and its implications for wound healing. J Altern Complement Med 2003; 9:267-273.
39. Soneja A, Drews M, Malinski T. Role of nitric oxide, nitroxidative and oxidative stress in wound healing. Pharmacol Rep 2005; 57 Suppl:108-119.
40. Schafer M, Werner S. Oxidative stress in normal and impaired wound repair. Pharmacol Res 2008; 58:165-171.
41. Hollnagel A, Kroh LW. Degradation of oligosaccharides in nonenzymatic browning by formation of alpha-dicarbonyl compounds via a "peeling off" mechanism. J Agric Food Chem 2000; 48:6219-6226.
42. Arena E, Ballistreri G, Tomaselli F, Fallico B. Survey of 1,2-dicarbonyl compounds in commercial honey of different floral origin. J Food Sci 2011; 76:C1203-1210.
43. Marshall SM, Schneider KR, Cisneros KV, Gu L. Determination of antioxidant capacities, alpha-dicarbonyls, and phenolic phytochemicals in Florida varietal honeys using HPLC-DAD-ESI-MS(n.). J Agric Food Chem 2014; 62:8623-8631.
44. Ortwerth BJ, James H, Simpson G, Linetsky M. The generation of superoxide anions in glycation reactions with sugars, osones, and 3-deoxyosones. Biochem Biophys Res Commun 1998; 245:161-165.
45. Safii SH, Tompkins GR, Duncan WJ. Periodontal Application of Manuka Honey: Antimicrobial and Demineralising Effects In Vitro. Int J Dent 2017; 2017:9874535.
46. Kwakman PH, Van den Akker JP, Guclu Aet al. Medical-grade honey kills antibiotic-resistant bacteria in vitro and eradicates skin colonization. Clin Infect Dis 2008; 46:1677-1682.
47. Kang JH. Oxidative damage of DNA induced by methylglyoxal in vitro. Toxicol Lett 2003; 145:181-187.
48. Ruckriemen J, Hohmann C, Hellwig M, Henle T. Unique fluorescence and high-

molecular weight characteristics of protein isolates from manuka honey (Leptospermum scoparium). Food Res Int 2017; 99:469-475.

49. Shangari N, Bruce WR, Poon R, O'Brien PJ. Toxicity of glyoxals--role of oxidative stress, metabolic detoxification and thiamine deficiency. Biochemical Society transactions 2003; 31:1390-1393.

3

CHAPTER 4

-

Histopathology and inflammatory features of chronically discharging open mastoid cavities

Darius Henatsch, Sultan Alsulami, Adriaan M. Duijvestijn, Jack P. Cleutjens, Carine J. Peutz-Kootstra, Robert J. Stokroos

JAMA Otolaryngology - Head and Neck Surgery. 2018 Mar 1;144(3):211-217

Abstract

Importance: Many patients with an open radical mastoid cavity experience therapy-resistant otorrhea. Little is known about the underlying histopathological substrate of unstable cavities and the correlation with treatment failure.

Objective: To study the histopathological and inflammatory features of chronically discharging open radical mastoid cavities and the influence of different treatments.

Design, setting and participants: This secondary analysis of a randomized clinical trial was a histopathology study of tissue samples of a cohort of 30 patients with a chronically discharging open mastoid cavity. Samples were taken from the cavity of patients, which were treated with either honey gel or conventional eardrops in a tertiary center between 2012 and 2013. Tissue staining was performed in May 2014; final computer analysis/correlation studies were performed in June 2016.

Main outcomes and measures: Differences of epithelial tissue coverage, infiltration of T cells (CD3, CD4, CD8) and macrophage (CD68, isoenzyme nitric oxide synthase, arginase 1) (sub-)populations, infection status, and the correlation with clinical presentation.

Results: There were 30 patients (24 [80%] male; mean [SD] age, 59 [14] years). Cavities were covered with either stratified squamous (keratinized) epithelium (n = 10), respiratory columnar epithelium (n = 9), or granulation tissue (n = 10). The presence of respiratory epithelium was associated with lower treatment success (posttreatment VAS improvement of 3.1 [95% CI, 0.5 to 5.8] for discomfort and 3.6 [95% CI, 0.2 to 6.9] for otorrhea in the group with granulation tissue coverage vs 4.9 [95% CI, 0.2 to 9.6] and 5.8 [95% CI, −0.1 to 11.6] in the group with squamous [keratinized] epithelium coverage and 1.4 [95% CI, −1.2 to 4.1] and 2.5 [95% CI, −1.3 to 6.2] in the group with respiratory columnar epithelium coverage). In all 3 tissue types of cavity-covering tissues, T-cell infiltrates consisted of helper T cells and cytotoxic T cells, together with a lower number of macrophages. The immunopositivity for isoenzyme nitric oxide synthase and arginase 1 was high and not restricted to a macrophage subpopulation, but seen in various cell types. Inflammatory infiltrations varied strongly in all 3 tissue modalities.

Conclusions and relevance: Discharging open mastoid cavities can be classified histologically into 3 different types, based on their coverage: squamous epithelium, respiratory epithelium, or granulation tissue. Treatment is less successful in cavities covered with respiratory epithelium, possibly explained by the status of bacterial infection and local immunological differences.

Introduction

Canal-wall-down mastoid surgery is a common procedure to manage cholesteatoma and chronic otitis media[1,2]. Despite a low rate of recurrence and satisfactory results[3,4], more than 20% of patients with an open mastoid cavity suffer from intermitted or continuous otorrhea[5,6], which is often resistant to therapy[7]. It seems that a major problem of unstable cavities is insufficient (re)epithelialization[8], which is favored by local conditions, unfavorable cavity shape, and size[9,10] and host factors[11]. Recently we presented promising results with the topical treatment of chronically discharging open mastoid cavities with honey gel, which led to less discomfort, otorrhea, inflammation and infection than conventional eardrops[12]. In other wounds, it was already shown that honey treatment stimulates better wound healing, wound debridement and epithelialization[13-16]. Chronically discharging cavities resemble chronic wounds by remaining in an uncoordinated, self-sustaining state of inflammation[17]. In these wounds, an abundance of pro-inflammatory macrophages seems to hamper wound progression and healing[18]. This type of macrophage outbalances wound-healing macrophages and is stimulated by T-helper 1 cells[19]. Honey has an immune-modulatory effect[20-22], and could contribute to a better wound healing on this cellular level.

Despite different treatment approaches, little is known about the underlying histopathological substrate of unstable cavities. Therefore, in this study we present the histological results of biopsies taken before and after treatment, during a 12-week clinical study, in which chronically discharging open radical mastoid cavities were treated with either medical honey or conventional eardrops, as published elsewhere[12]. We aimed to investigate the histopathology of unstable cavities and their correlation with clinical presentation and treatment response, with a special focus on pro-inflammatory immune mechanisms by T-cell and macrophage subsets.

Methods

Study population

Histological samples were obtained from 30 patients who were enrolled in a clinical study as previously described[12]. The study was approved by the ethics committee of Maastricht University Medical Center and all patients gave their written informed con- sent prior to the start of the study. Briefly, patients with a chronically infected open mastoid cavity were recruited from April 2012 until September 2013 in a single-center, randomized controlled, double-dose trial, conducted at the Maastricht University Medical Center+, The Netherlands[12]. After inclusion a swab sample and a biopsy were taken from the cavity and patients were treated with either Terra-Cortril Polymyxin B eardrops (hydrocortisone, oxytetracycline, and polymyxin B) for 1 week, or the medical honey gel NasuMel (Revamil honey mixed with water and hydroxyethylcellulose). Treatment was repeated after 4 weeks. A second swab and biopsy was taken at 8 weeks from start, and patients filled in a visual analogue scale (VAS) about their cavity problems.

Biopsies

The site of biopsy was topically anesthetized with lidocaine 10% for 10 minutes and a small tissue sample of several millimeters was taken with a Blakesley forceps. The biopsy was taken from the part of the cavity with macroscopically most signs of infection, that is, granulation tissue, pus or erythema. When no signs of infections were present, a random biopsy was taken.

Immunohistochemical staining and evaluation

Tissue specimens were immediately fixed in 4% buffered formaldehyde and processed by regular histological procedures. Biopsies were paraffin embedded and sectioned in 4-μm slices. Parallel sections were stained with hematoxilin-eosin and Periodic acid–Schiff. Tissue sections were scored for extent of epithelial tissue coverage and presence of inflammation by two independent observers (C.J.P.-K. and D.H). Tissue was obtained from the Maastricht Pathology Tissue Collection. Collection, storage and use of tissue and patient data were performed in agreement with the Code for Proper Secondary Use of Human Tissue in the Netherlands (http://www.federa.org). Sections were also immunohistochemically stained with monoclonal antibodies defining T cells and macrophages: CD3 (total T cells), polyclonal rabbit anti-human-CD3 (Dako); CD4 (helper T cells), monoclonal mouse anti-human-CD4 (Dako); CD8 (cytotoxic T cells), monoclonal mouse anti-human-CD8 (Dako); CD68 (macrophages), monoclonal mouse anti-human-CD68 (Dako); isoenzym nitric oxide synthase (iNOS) and Arginase-1 (Arg-1) (wound healing markers in macrophage subsets), polyclonal rabbit anti-human-iNOS antibody (Abcam) and polyclonal rabbit anti-human-Arg-1 (provided by P. van Dijk, Maastricht University, Netherlands), respectively. Computer-assisted color imaging analysis was performed using the histomorphometry software Leica Qwin, version 3. Macrophage and T-cell content was expressed as percentage of positive cells of total tissue area.

Statistical analysis

Normally distributed continuous data were compared using the paired *t* test and effect size was expressed using Cohen's *d*. The exact binomial test in the R software package[23] was used to calculate the probability of a positive test result of microbiological swabs. Mean difference data were reported, including a 95% CI.

Results

Clinical outcome

A previously reported[12] cohort of 30 patients (24 male) with a mean (SD) age of 59 (14) years was used for this study. Patients had an open cavity for a mean of 19 years (range, 1-57 years), and 8 (27%) had continuous problems with it since they underwent surgery. Eighteen were treated with honey gel and 12 with eardrops. Topical honey treatment led to less discomfort and otorrhea (measured by VAS) and a macroscopically improvement of cavity inflammation, compared with eardrops[12]. Of all cavities, 8 (27%) were colonized with *Pseudomonas* species, 6 (20%) with *Staphylococcus aureus* and 7 (23%) with other species. The incidence of pathological bacterial infection was reduced by treatment for 4 (23%) in the honey group, compared to 3 (30%) in the eardrops group[12].

Histopathological analysis of the chronically discharging open mastoid cavity shows different subtypes

Thirty biopsies were taken before patients were treated with either honey gel or eardrops. Twenty-nine tissue fragments were available for histological analysis. Three different types of epithelial tissue coverage were found in the biopsies: coverage with either stratified squamous (keratinized) epithelium, respiratory (ciliated) columnar epithelium or granulation tissue without epithelial coverage

(Fig. 1). In 10 patients (34%), tissue fragments were covered with stratified squamous (keratinized) epithelium, in some with hyperkeratosis. In all these biopsies, inflammation was present, with mainly lymphoplasmacellular and plasmacytoid infiltration with neutrophils. In some samples, eosinophils and macrophages were seen as well.

In 6 patients (21%), tissue was additionally focally lined with (pseudo-stratified) ciliated columnar epithelium, which was associated with more extensive inflammatory infiltration including lymphocytes, plasma cells and neutrophils. In 3 tissue fragments (10%), only ciliated columnar epithelium was present, with in subepithelial layers fibrosis with prominent active, chronic inflammatory infiltration, with lymphocytes, polymorphonuclear neutrophils, macrophages and some eosinophilic granulocytes.

Ten tissue fragments (34.5%) of cavity coverage consisted of granulation tissue with different degrees of fibrosis and active, chronic inflammatory infiltration with lymphocytes, eosinophils, polymorphonucelear neutrophils, plasma cells and occasionally macrophages and giant cells. Inflammation was accompanied with vascular proliferation in most samples. One tissue sample was damaged and could not be analyzed.

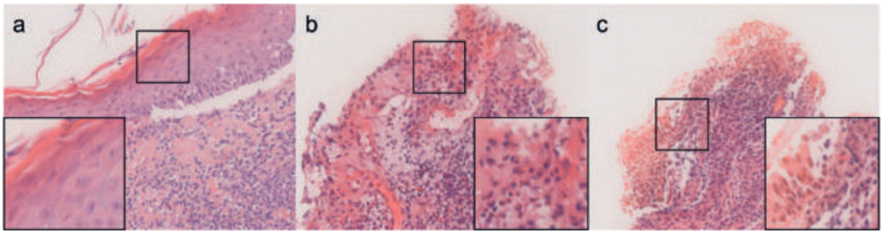


Fig. 1. Photomicrographs of 3 different tissue specimens taken prior to treatment. Tissue is either covered with stratified (keratinized) epithelium (a), consists of granulation tissue coverage (b) or is covered with ciliated columnar epithelium (c). Main image in each panel is original magnification ×20; large insets are high-power micrographs showing a 4× enhancement of the small inset in each panel. Staining is hematoxylin-eosin and periodic acid–Schiff.

Histological features correlate with reported clinical outcome

Patients filled in a VAS about discomfort and otorrhea before the treatment and 4 weeks after the second treatment (8 weeks later), when the second biopsy was taken. The histological differences of an unstable cavity seemed to be associated with the outcome of successful treatment. Patients with a cavity with a (partial) coverage of granulation tissue or with a coverage of stratified squamous (keratinized) epithelium prior to treatment showed a much better VAS improvement (treatment response), compared with patients with a cavity (partially) covered with respiratory columnar epithelium (Fig. 2). This response was irrespective of the treatment modality. The improvement of VAS after treatment was 3.1 (95% CI, 0.5 to 5.8) for discomfort and 3.6 (95% CI, 0.2 to 6.9) for otorrhea in the group with granulation tissue coverage prior to treatment, compared to respectively, 4.9 (95% CI, 0.2 to 9.6) and 5.8 (95% CI, -0.1 to 11.6) in the group with squamous (keratinized) epithelium coverage and 1.4 (95% CI, -1.2 to 4.1) and 2.5 (95% CI, -1.3 to 6.2) in the group with respiratory columnar epithelium coverage.

In addition, there was a nonsignificant finding that a microbiological swab sample that was positive for pathologic bacteria was possibly associated with a cavity (partially) covered with respiratory epithelium. In patients with (partial) coverage with granulation tissue a positive swab with pathologic bacteria was found in 50% (n = 5; 95% CI, 19%-81%), compared to 60% (n = 6; 95% CI, 26%-88%) in the group with squamous (keratinized) epithelium coverage and 78% (n = 7; 95% CI, 40%-97%) in the group of patients with (partial) coverage with respiratory columnar epithelium. However, no association was seen with gram-positive or gram-negative species.

The (partial) cavity coverage changed considerably after treatment. In 10 patients, biopsies showed granulation tissue coverage before treatment. After treatment in this group 4 biopsies changed to squamous (keratinized) epithelium coverage. In the group in which biopsies consisted of respiratory columnar epithelium coverage (10 patients), this coverage changed to squamous (keratinized) epithelium in 7 patients. In patients, who had coverage with squamous (keratinized) epithelium prior to treatment (10 patients), this coverage stayed unchanged in 6 patients and changed to coverage consisting of granulation tissue in 2 patients (2 patients of this group were excluded). Thus, regardless of treatment, a high proportion of granulation and respiratory epithelium coverage altered to coverage with squamous epithelium.

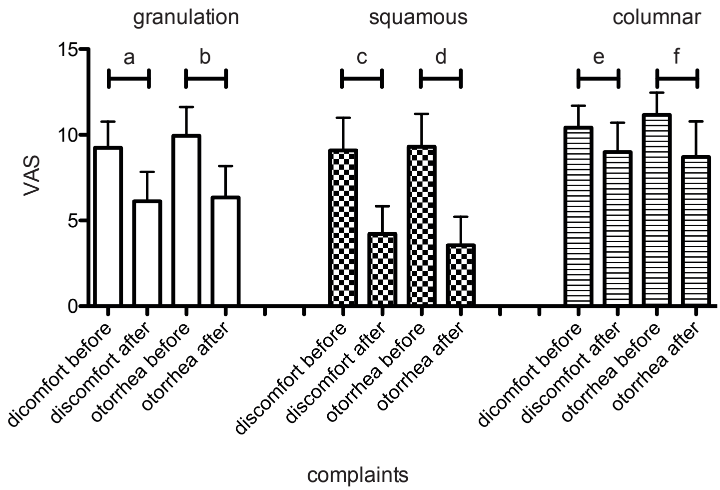


Fig. 2. Patient's visual analog scale (VAS) of discomfort and otorrhea before and after treatment in correlation to histology features of biopsy specimens obtained prior to treatment.
The VAS of discomfort and otorrhea was measured before treatment (at the same time the first biopsy was obtained) and compared with the VAS after treatment (8 weeks later). Characters indicate effect size (Cohen *d*): a=0.64; b=0.68; c=1.04; d=1.21; e=0.33; f=0.5. Bars indicate mean, and error bars, standard deviation.

Immunohistochemical features of the chronically discharging open mastoid cavity

T-cell and macrophage infiltration

In all 3 epithelial tissue modalities, varying intensities of CD3-positive T cells were present, mainly in underlying granulation tissue. Subtyping of this population showed that helper T cells (CD4 positive) and cytotoxic T cells (CD8 positive) are present in equal numbers within the biopsies. We found no skewing of the immune response and no specific association of T-cell phenotype within either of the histological subtypes (Fig. 3). Furthermore, we found relatively low numbers of macrophages (CD68 positive) in the biopsies (Fig. 3). The positive staining of CD68-positive macrophages was not associated with histological subtypes, although there seemed to be a higher abundance of macrophages in areas of fibrosis.

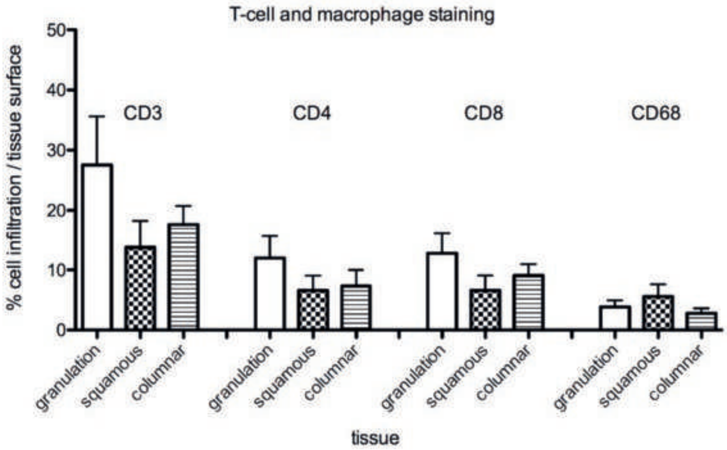


Fig. 3. Tissue-dependent immunopositivity for T cells (CD3), T-cell subtypes (CD3 and CD4), and macrophages (CD68). Bars indicate mean, and error bars, standard deviation.

iNOS and Arg-1 positivity

A constitutively iNOS immunopositivity was seen diffusely and intensively in squamous and respiratory epithelium, as well as a moderate to low intensity for endothelial cells, smooth muscle cells and fibroblasts. A moderate to strong positivity was seen for macrophages, without specificity for a macrophage subpopulation and other immune cells. Overall, Arg-1 expression followed a similar pattern, and almost seemed to be co-expressed with iNOS in epithelial cells, inflammatory cells and mesenchymal cells (Fig. 4). Here as well, no specific macrophage subtype (pro-inflammatory or wound-healing) was identifiable. Because of the strong expression pattern of both iNOS and Arg-1, we could neither quantitatively discern differences in expression levels between histological subtypes nor between treatment groups.

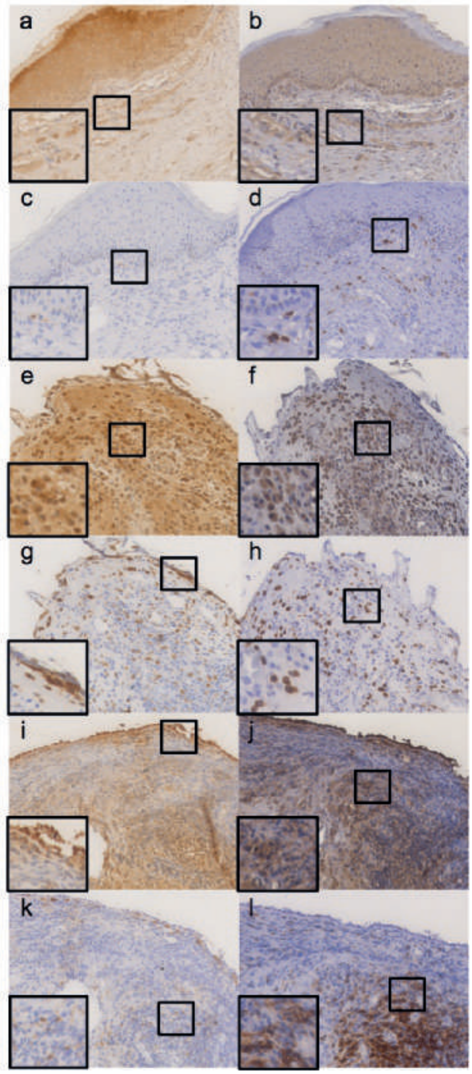


Fig. 4. Photomicrographs of 3 different tissue specimens. Tissue covered with stratified squamous epithelium stained for Arg-1 (a), iNOS (b), CD68 (macrophages) (c) and CD3 (T cells) (d). Granulation tissue stained for Arg-1 (e), iNOS (f), macrophages (g) and T-cells (h). Tissue covered with ciliated columnar epithelium stained for Arg-1 (i), iNOS (j), macrophages (k) and T-cells (l). Main image in each panel is original magnification ×20; large insets are high-power micrographs showing a 4× enhancement of the small inset in each panel.

Discussion

In this histopathological study, we show that chronically discharging open mastoid cavities are (partially) covered with 3 different types of tissue, either stratified squamous (keratinized) epithelium, respiratory (ciliated) columnar epithelium or granulation tissue without an epithelial coverage. Treatment of patient with respiratory epithelium coverage seems to be less successful, likely as a result of bacterial infection. All 3 types of biopsies with different epithelial coverage show T-cell infiltration, consisting of more or less equal numbers of helper and cytotoxic T cells. Also, low numbers of macrophages are present in the 3 tissue types. The 3 types of tissue coverage show a high immunopositivity for iNOS and Arg-1 in almost all macrophages. Therefore, no indication of macrophage skewing towards a proinflammatory or anti-inflammatory (wound-healing) phenotype of the immune response was detected.

Until now, little is known about the underlying histopathological features of an unstable, chronically discharging radical mastoid cavity. The postoperative and postinflammatory healing of an open cavity, can only partly be compared with wound healing elsewhere. In a cavity, skin has to grow on bare bone, a difficult process[24], which is further hindered by a humid environment, exudation, infection and keratin debris[25]. Normal wound healing consists of a well-structured process of inflammation, proliferation and remodeling[17], in which reepithelialization is essential for wound closure. The latter is closely associated with granulation tissue formation[26]. The moist environment of an open cavity favors granulation tissue growth[24], which again interferes with epithelialization[27].

We show in this study that the coverage of an unstable open cavity consists of 3 different tissue types, that is, stratified squamous (keratinized) epithelium, respiratory columnar epithelium and granulation tissue. Importantly, this histological classification at the moment of clinical interference is associated with treatment success. A possible explanation for this finding is that granulation tissue formation and reepithelialization are spatially and temporally closely correlated in wound healing[26] and a better treatment response is reasonable. We hypothesize that respiratory epithelium in contrast followed a process of metaplasia, rather than normal epithelial differentiation.

Honey and conventional eardrops were both effective in treating unstable cavities, as shown earlier[12]. The coverage of unstable cavities changed profoundly as well. Most cavities that had been covered with granulation tissue or respiratory epithelium prior to treatment changed to coverage with squamous epithelium after treatment. This was regardless of the treatment modality. We noticed baseline differences in the different sorts of cavity coverage, with more cavities covered with granulation tissue in the honey group, which was also seen after treatment. Possible explanations for histological differences that were seen in our study are the influence of chronic bacterial infection and local immune reactions. The presence of endotoxins, cell wall products from gram-negative bacteria, has been shown to lead to a prolongation of wound healing by means of an impairment of epithelialization[28]. We showed earlier that approximately 30% of cavities were colonized with *Pseudomonas* species[12], which are gram-negative. However, we could not find a correlation with differences in coverage of the cavity and gram-negative species, but we found a tendency towards higher swab positivity in the patient group with respiratory epithelial cavity coverage. Bacterial infection[29,30] and inflammation[31] cause oxidative stress that impairs fibroblasts and hinders healing[32].

An important molecule during inflammation, wound healing and reepithelialization is nitric oxide (NO)[33]. Isoenzyme nitric oxide synthase synthetizes NO, together with citrulline from arginine. Isoenzyme nitric oxide synthase competes for this substrate with another enzyme called arginase, which converts arginine into ornithine and urea[34]. Isoenzyme nitric oxide synthase and Arg-1 regulation seems to play an important role in normal[35] and altered wound healing[36,37], and the dysregulation of both may play a key role in impaired wound healing and granulation tissue formation[38]. We chose iNOS and Arg-1 as accepted macrophage markers[19,37,39] to differentiate between a classically activated (proinflammatory) and wound healing subtype. A treatment-dependent switch in subtype populations could be responsible for wound healing progression[18,19]. Contrary to these expectations, we observed a high immunopositivity for both, iNOS and Arg-1 in various cell types, without specificity for macrophages. Other studies showed a high expression for iNOS in epithelial, endothelial, and smooth muscle cells, as well as macrophages, fibroblasts and polymorphonuclear neutrophils in wounds[35]. In infected chronic wounds, increased Arg-1 levels are also found[36]. Different sources for this enhanced expression are reported, as polymorphonuclear neutrophils[35], wound margin keratinocytes[38], fibroblasts[40] and macrophages[37]. An altered high iNOS[37] and Arg-1[38] expression is associated with chronic wound healing conditions. The enhanced expression of both, iNOS and Arg-1 in non-healing open mastoid cavities indicate an important role of NO metabolism in this chronic healing problem.

This is very important because different medications are interacting with the NO metabolism during wound healing. As discussed earlier, honey seems to be an

4

effective treatment of unstable cavities. It was shown in different models, that honey has a positive influence on NO metabolism[41], with a net anti-inflammatory effect[42,43]. In contrast, there are indications that corticosteroid application has a negative effect on NO metabolism in the skin[44], and during inflammation[45]. This knowledge can influence future treatment strategies. In the Netherlands common treatments of chronically discharging cavities are repeated debridement, gentian violet application, local cauterization and boric acid powder treatment, which has a good proven effect in otitis[46,47].

Based on the results of this study it is not possible to advise 1 specific treatment, but we can argue that coverage of a mastoid cavity with respiratory columnar epithelium is associated with treatment failure and possibly bacterial infection and necessitates a long-lasting and more repetitive treatment strategy or even revision surgery.

Furthermore, novel drugs, such as NO-delivering medications that have a positive effect on wound healing[48,49], could play an important role in the disturbed NO metabolism in the nonhealing cavity.

Limitations

To our knowledge, this is the first study that shows the histological differences of cavity coverage and the underlying immunological substrate in patients with an active discharging open mastoid cavity. This study is in line with earlier postmortem studies, which also showed that unstable cavities were predominantly covered with stratified squamous keratinized epithelium with fibrosis and inflammatory infiltrate in subepithelial layers. There, coverage with respiratory epithelium was accompanied with a more intense inflammatory infiltrate[50,51].

Conclusion

In patients with chronically discharging open mastoid cavities, typing of the cavity coverage is important for treatment expectations. Histologically, 3 different types of coverage can be discriminated, either stratified squamous (keratinized) epithelium, respiratory columnar epithelium or granulation tissue. Respiratory columnar epithelium is associated with treatment failure and with bacterial infection.

References

1. Rombout J, Pauw BK. Radical revision mastoidectomy for chronic otitis media without cholesteatoma: the relevance of excenteration of all rest cells. *J Laryngol Otol*. 1999;113(8):710-713.
2. Mukherjee P, Saunders N, Liu R, Fagan P. Long-term outcome of modified radical mastoidectomy. *J Laryngol Otol*. 2004;118(8):612-616.
3. Aslan Felek S, Islam A, Celik H, Demirci M, Samim E, Kose SK. The functional and anatomical results of the canal wall down tympanoplasty in extensive cholesteatoma. *Acta Otolaryngol*. 2009;129(12):1388-1394.
4. Kos MI, Castrillon R, Montandon P, Guyot JP. Anatomic and functional long-term results of canal wall-down mastoidectomy. *Ann Otol Rhinol Laryngol*. 2004;113(11):872-876.
5. Beales PH. Complications following obliterative mastoid operations. *Arch Otolaryngol*. 1969;89(1):196-198.
6. Palva T. Surgical treatment of chronic middle ear disease. III. Revisions after tympanomastoid surgery. *Acta Otolaryngol*. 1988;105(1-2):82-89.
7. Ishimoto S, Ito K, Sasaki T, Shinogami M, Kaga K. Total middle ear reconstructive surgery for the radicalized ear. *Otol Neurotol*. 2002;23(3):262-266.
8. Premachandra DJ, Woodward B, Milton CM, Sergeant RJ, Fabre JW. Treatment of chronic mastoiditis by grafting of mastoid cavities with autologous epithelial layers generated by in vitro culture of buccal epithelium. *J Laryngol Otol*. 1991;105(6):413-416.
9. Wormald PJ, Nilssen EL. The facial ridge and the discharging mastoid cavity. *Laryngoscope*. 1998;108(1 Pt 1):92-96.
10. Sade J, Weinberg J, Berco E, Brown M, Halevy A. The marsupialized (radical) mastoid. *J Laryngol Otol*. 1982;96(10):869-875.
11. Gluth MB, Metrailer AM, Dornhoffer JL, Moore PC. Patterns of failure in canal wall down mastoidectomy cavity instability. *Otol Neurotol*. 2012;33(6):998-1001.
12. Henatsch D, Wesseling F, Briede JJ, Stokroos RJ. Treatment of chronically infected open mastoid cavities with medical honey: a randomized controlled trial. *Otol Neurotol*. 2015;36(5):782-787.
13. Molan PC. The evidence supporting the use of honey as a wound dressing. *Int J Low Extrem Wounds*. 2006;5(1):40-54.
14. Molan PC. Potential of honey in the treatment of wounds and burns. *American journal of clinical dermatology*. 2001;2(1):13-19.
15. Jull AB, Rodgers A, Walker N. Honey as a topical treatment for wounds. *Cochrane Database Syst Rev*. 2008(4):CD005083.
16. Brolmann FE, Ubbink DT, Nelson EA, Munte K, van der Horst CM, Vermeulen H. Evidence-based decisions for local and systemic wound care. *The British journal of surgery*. 2012;99(9):1172-1183.
17. Schreml S, Szeimies RM, Prantl L, Landthaler M, Babilas P. Wound healing in the 21st century. *J Am Acad Dermatol*. 2010;63(5):866-881.
18. Sindrilaru A, Peters T, Wieschalka S, et al. An unrestrained proinflammatory M1

4

macrophage population induced by iron impairs wound healing in humans and mice. *J Clin Invest*. 2011;121(3):985-997.
19. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nature reviews Immunology*. 2008;8(12):958-969.
20. Tonks A, Cooper RA, Price AJ, Molan PC, Jones KP. Stimulation of TNF-alpha release in monocytes by honey. *Cytokine*. 2001;14(4):240-242.
21. Mesaik MA, Azim MK, Mohiuddin S. Honey modulates oxidative burst of professional phagocytes. *Phytother Res*. 2008;22(10):1404-1408.
22. Tonks AJ, Cooper RA, Jones KP, Blair S, Parton J, Tonks A. Honey stimulates inflammatory cytokine production from monocytes. *Cytokine*. 2003;21(5):242-247.
23. The R [online software], version 3.2.3, Wooden Christmas-Tree, Foundation for Statistical Computing, i386-w64-mingw32/i386 (32-bit), https://cran.r-project.org/src/base/R-3/. 2015.
24. Chhapola S, Matta I. Mastoid obliteration versus open cavity: a comparative study. *Indian journal of otolaryngology and head and neck surgery : official publication of the Association of Otolaryngologists of India*. 2014;66(Suppl 1):207-213.
25. Jothiramalingam S, Kumar D, Kumar P, Sasindran V, Kumar N. Atticoantral disease — revisited. *Indian Journal of Otolaryngology and Head & Neck Surgery*. 2007;59(3):203-206.
26. Goldman R. Growth factors and chronic wound healing: past, present, and future. *Adv Skin Wound Care*. 2004;17(1):24-35.
27. Somers T, Duinslaeger L, Delaey B, et al. Stimulation of epithelial healing in chronic postoperative otorrhea using lyophilized cultured keratinocyte lysates. *Am J Otol*. 1997;18(6):702-706.
28. Riedel K, Ryssel H, Koellensperger E, Germann G, Kremer T. [Pathogenesis of chronic wounds]. *Chirurg*. 2008;79(6):526-534.
29. Takoudes TG, Haddad J, Jr. Hydrogen peroxide in acute otitis media in guinea pigs. *Laryngoscope*. 1997;107(2):206-210.
30. Takoudes TG, Haddad J, Jr. Free radical production by antibiotic-killed bacteria in the guinea pig middle ear. *Laryngoscope*. 2001;111(2):283-289.
31. Schafer M, Werner S. Oxidative stress in normal and impaired wound repair. *Pharmacol Res*. 2008;58(2):165-171.
32. Moseley R, Stewart JE, Stephens P, Waddington RJ, Thomas DW. Extracellular matrix metabolites as potential biomarkers of disease activity in wound fluid: lessons learned from other inflammatory diseases? *Br J Dermatol*. 2004;150(3):401-413.
33. Luo JD, Chen AF. Nitric oxide: a newly discovered function on wound healing. *Acta pharmacologica Sinica*. 2005;26(3):259-264.
34. Childress B, Stechmiller JK, Schultz GS. Arginine metabolites in wound fluids from pressure ulcers: a pilot study. *Biological research for nursing*. 2008;10(2):87-92.
35. Debats IB, Wolfs TG, Gotoh T, Cleutjens JP, Peutz-Kootstra CJ, van der Hulst RR. Role of arginine in superficial wound healing in man. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society*. 2009;21(3-4):175-183.
36. Debats IB, Booi D, Deutz NE, Buurman WA, Boeckx WD, van der Hulst RR. Infected chronic wounds show different local and systemic arginine conversion compared

with acute wounds. *J Surg Res*. 2006;134(2):205-214.
37. Campbell L, Saville CR, Murray PJ, Cruickshank SM, Hardman MJ. Local arginase 1 activity is required for cutaneous wound healing. *J Invest Dermatol*. 2013;133(10):2461-2470.
38. Kampfer H, Pfeilschifter J, Frank S. Expression and activity of arginase isoenzymes during normal and diabetes-impaired skin repair. *J Invest Dermatol*. 2003;121(6):1544-1551.
39. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity*. 2010;32(5):593-604.
40. Witte MB, Barbul A, Schick MA, Vogt N, Becker HD. Upregulation of arginase expression in wound-derived fibroblasts. *J Surg Res*. 2002;105(1):35-42.
41. Kassim M, Yusoff KM, Ong G, Sekaran S, Yusof MY, Mansor M. Gelam honey inhibits lipopolysaccharide-induced endotoxemia in rats through the induction of heme oxygenase-1 and the inhibition of cytokines, nitric oxide, and high-mobility group protein B1. *Fitoterapia*. 2012;83(6):1054-1059.
42. Owoyele BV, Adenekan OT, Soladoye AO. Effects of honey on inflammation and nitric oxide production in Wistar rats. *Zhong Xi Yi Jie He Xue Bao*. 2011;9(4):447-452.
43. Kassim M, Achoui M, Mansor M, Yusoff KM. The inhibitory effects of Gelam honey and its extracts on nitric oxide and prostaglandin E(2) in inflammatory tissues. *Fitoterapia*. 2010;81(8):1196-1201.
44. Abraham A, Roga G. Topical steroid-damaged skin. *Indian J Dermatol*. 2014;59(5):456-459.
45. Linehan JD, Kolios G, Valatas V, Robertson DA, Westwick J. Effect of corticosteroids on nitric oxide production in inflammatory bowel disease: are leukocytes the site of action? *Am J Physiol Gastrointest Liver Physiol*. 2005;288(2):G261-267.
46. Amani S, Moeini M. Comparison of Boric Acid and Combination Drug of Polymyxin, Neomycin and Hydrocortisone (polymyxin NH) in the Treatment of Acute Otitis Externa. *Journal of clinical and diagnostic research : JCDR*. 2016;10(7):MC01-04.
47. Loock JW. A randomised controlled trial of active chronic otitis media comparing courses of eardrops versus one-off topical treatments suitable for primary, secondary and tertiary healthcare settings. *Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery*. 2012;37(4):261-270.
48. Weller R, Finnen MJ. The effects of topical treatment with acidified nitrite on wound healing in normal and diabetic mice. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society*. 2006;15(4):395-399.
49. Amadeu TP, Seabra AB, de Oliveira MG, Monte-Alto-Costa A. Nitric oxide donor improves healing if applied on inflammatory and proliferative phase. *J Surg Res*. 2008;149(1):84-93.
50. Youngs R. The histopathology of mastoidectomy cavities, with particular reference to persistent disease leading to chronic otorrhoea. *Clinical otolaryngology and allied sciences*. 1992;17(6):505-510.
51. Youngs R. Temporal bone histopathology of open mastoidectomy cavities. *J Laryngol Otol*. 1993;107(6):569-573.

CHAPTER 5

-

Treatment of chronically infected open mastoid cavities with medical honey: a randomized controlled trial

Darius Henatsch, Frederik Wesseling, Jacob J. Briedé, Robert J. Stokroos

Otology and Neurotology 2015 Jun;36(5):782-787

Abstract

Objective: To investigate the efficacy of medical honey as topical treatment of chronically discharging open mastoid cavities in comparison with conventional eardrops.

Study design: Single center, prospective, randomized controlled, double-dose trial of 12 weeks.

Patients and intervention: Twenty-eight patients diagnosed as having a chronically discharging open mastoid cavity underwent medical honey gel (intervention) or conventional eardrops (control) treatment. Treatment interventions were repeated after 4 weeks.

Main outcome measures: Visual analogue scale of ear complaints, cavity inflammation, and bacterial infection.

Results: Most patients had a cavity with localized granulation. After treatment, inflammation score decreased in both groups ($p < 0.05$), with more pronounced inflammation-free cavities in the honey group. Honey treatment resulted in less discomfort ($p < 0.001$) and otorrhea ($p < 0.001$), even after correction for additional medication use ($p < 0.05$, $p < 0.01$). This decrease was not seen in the control group. Pain and itching did not change on treatment. Most cavities were infected with *Pseudomonas* species and *Staphylococcus aureus*. After treatment, a 23% increase of negative culture was seen with honey compared with 30% in the control group (nonsignificant). No serious adverse reactions were found.

Conclusion: Medical honey gel is a safe alternative treatment option for patients with a chronically discharging open mastoid cavity and beneficial in reducing discomfort, otorrhea and inflammation with a bactericidal effect.

Introduction

The aim of middle ear surgery for chronic otitis media and cholesteatoma is to create a disease-free and stable ear[1]. The canal wall down technique, with the formation of an open mastoid cavity, allows complete disease visualization and is in most patients a single-stage procedure[2,3]. In addition, the recurrence rate is less than 10% with satisfactory hearing results[4]. A major disadvantage of an open cavity is the need for periodic mastoid cavity cleaning[3]. More than 20% of patients with an open mastoid cavity continue to have intermittent or persistent otorrhea even with regular aural toilet and topical medication[5]. Ear complaints can persist up to 30 years after surgery[6], with a high socioeconomic impact[7]. Chronic cavity inflammations are caused by limited self-cleansing, insufficient aeration, and failure of epithelialization[1,6]. Histologically, cavity lining consists of stratified, squamous, keratinizing epithelium with underlying fibrosis, partly with inflammatory infiltration[5]. Unstable cavities show many characteristics of chronic wounds, by remaining in an uncoordinated, self-sustaining state of inflammation[8]. In these wounds, honey was rediscovered as an alternative dressing. Honey stimulates wound healing, epithelialization, wound debridement and leads to less malodor[9-12].

No studies are performed so far, to evaluate honey as alternative treatment for unstable open mastoid cavities. The aim of this study was to investigate the efficacy of the medical honey product NasuMel (NM) as topical treatment of chronically discharging open mastoid cavities compared to Terra-Cortril Polymyxine B eardrops (TC).

Methods

Objective

The primary objectives of the study were patient visual analogue scale (VAS) of ear complaints, severity of cavity inflammation, and bacterial infection. Secondary objectives were need of patients' revisits and extra treatment in the outpatient clinic.

Study design

This study was designed as a single center, randomized controlled, double dose trial conducted at the Maastricht University Medical Center. Randomization was performed using previously prepared randomly numbered opaque envelopes. The study was approved by the ethical committee of the Maastricht University Medical Center in Maastricht (protocol no. 10-04-2011). All procedures were in accordance with the Helsinki Declaration.

Study medication

NasuMel gel (Bfactory Health Products BV, Rhenen, The Netherlands) contains more than 85% pure Revamil honey mixed with water and hydroxyethylcellulose. The gel was delivered in a 5-ml syringe with a sterile cannula for ear application. Revamil, the source honey of NM, has proven antibacterial capacity[13-16]. Terra-Cortril Polymyxine B eardrops (Pfizer BV, Capelle a/d IJssel, The Netherlands) contain hydrocortisone, oxytetracycline, and polymyxin B and are commonly used eardrops in the Netherlands.

Patients

Thirty patients with an infected open mastoid cavity were recruited from April 2012 until September 2013 in a consecutive manner. Patients ranging between 18 and 80 years were included when suffering from infection of an open mastoid cavity. *Infection* was defined as actual otorrhea, signs of discharge on pillow or clothes, pruritus, feeling of fullness, or signs of infection during otological examination. Exclusion criteria were other experimental treatments of the ear, chronic intake of immune modulating agents and/or antibiotics, having an allergy against honey, and incapacitated patients. All patients gave their written informed consent before the start of the study.

Intervention and outcome measures

All patients filled in a VAS about discomfort, otorrhea, pain and itching of their cavity. Afterwards the physician scored inflammation of the mastoid cavity using an adapted Merchant score[17] (Table 1) and took a bacterial swab of the cavity.

Table 1. Adapted Merchant Score

0	No complaints, no pus, or granulation tissue on examination
1	No otorrhea but subjective feeling of wetness in the ear
2	Otorrhea and otologic exam showing pus
3	Localized granulation tissue/pus
4	Extensive granulation tissue

In every patient, scoring was performed by the same investigator and an otologists, who were both blinded. In case of disagreement, the score was determined by joint agreement. Thereafter, patients were randomly allocated to the treatment, either NM or TC. For TC-treated patients, 3 droplets of TC were applied by the physician and the patients were instructed to continue the medication 3 times daily for 1 week. Patients were instructed to administer droplets in a lying position, with the affected ear upward and to remain in this position for about 3 to 5 minutes.

For NM-treated patients, NM was prewarmed to body temperature and injected until the cavity was completely filled. Afterwards, the cavity was sealed by an ear tampon, which was removed by the patient after 4 to 5 days. Patients were scheduled for a second visit after 4 weeks (Visit 2) for scoring and to repeat the treatment. After 8 weeks (Visit 3), scoring was repeated and again a bacterial swab was taken. After 12 weeks (Visit 4) patients were seen for the last time for scoring. All patients were instructed to avoid water contact and manipulation. Patients were asked to contact the outpatient clinic by phone for questions, adverse events or complaints of the cavity. In case of complaints as pain or otorrhea, patients were seen by the principal investigator within 3 days. Then a blinded physician of the study team decided if the patient needed medical treatment, outside the study medication protocol.

Statistical analysis

A chi-square test was used to compare binary data. Normal distributed continuous data were compared using the paired and unpaired *t* test. Mann-Whitney *U* test was used for unnormalized distributed data. Significance was determined to be at the confidence level of $p < 0.05$. Data are presented as mean ± standard deviation.

Results

Patient characteristics

A total of 30 patients were enrolled in the study. Eighteen patients were treated with NM, and 12 patients were treated with TC. Patient baseline characteristics and differences are shown in Table 2.

Table 2. Means and differences of baseline characteristics

	TC group	NM group	*p* value
Sex (male:female)	11:1	13:5	
Age (yr)	56 (±17)	60 (±12)	p=n.s.
Previous ear surgeries	2.0 (±0.9)	2.1 (±1.1)	p=n.s.
Years after surgery	19 (±16)	19 (±21)	p=n.s.
Days from last ENT visit	75 (±104)	72 (±69)	p=n.s.
Treated at last visit (%)	67	67	p=n.s.
Otorrhea (wk)*	39	44	p=n.s.
Ear complaints (mo)*	10	24	p=0.10
Ear medication use (mo)*	3	3	p=n.s.

ENT indicates ear, nose and throat; n.s., nonsignificant.
*Results are expressed as median.

Briefly, 80% of all patients were male and had an open cavity on average for 19 years, ranging from 1 to 57 years. Forty percent of patients had at least 1 revision surgery. Sixty-three percent of patients had continuous complaints (no more than 6 wk without ear complains) of which 25% continuous complaints since they underwent surgery. Three patients (two in the TC group) reported to be diagnosed with diabetes type II of which two were insulin dependent. The number of days between each scheduled study visit is shown in Fig. 1.

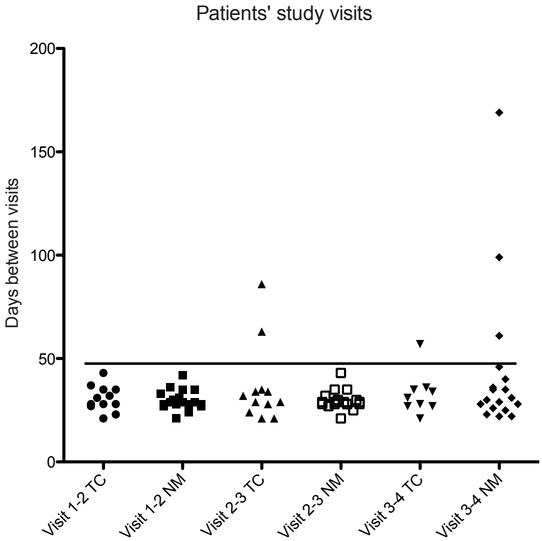


Fig. 1. Days between study visits.

Two patients in the control group were excluded from analysis because of a delay between the second visit and the third visit. Three patients in the NM group and one patient in the TC group were only evaluated until the third study visit because of a delay of the forth visit.

Primary outcome measures

After Visit 3, patients in the TC group reported a decrease of discomfort, but not otorrhea (Fig. 2A). On Visit 3 and 4, the NM group experienced a decrease of discomfort and otorrhea (Fig. 2B). In the TC group, no change in pain was reported, but a decrease in itching after Visit 4 (Fig. 2C). Baseline VAS of itching was lower in the NM group. Pain and itching of the NM group did not change during the study (Fig. 2D).

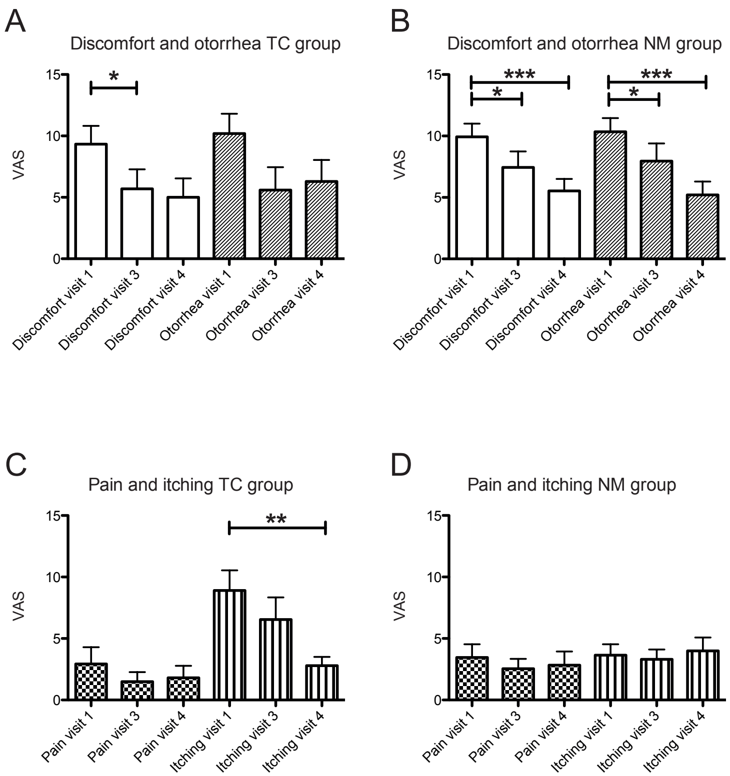
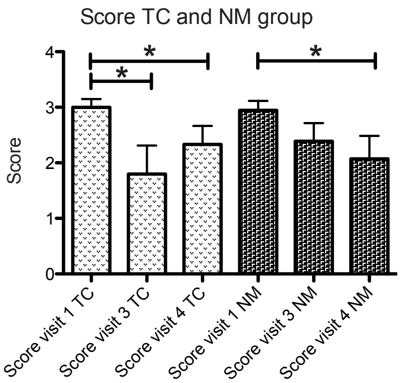


Fig. 2. Patients' VAS of discomfort, otorrhea, itching and pain (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

After correction for additional medication use, a decrease of discomfort and otorrhea in the NM group can still be found, which was not observed in the TC group. After correction, no changes were reported in both groups for pain and itching. Also, inflammation of the cavity was scored. During the baseline measurement, four patients had a score of 4, 20 patients a score of 3, four patients had a score of 2, and two patients a score of 1. In both groups, a decrease of inflammation was seen (Fig. 3). At Visit 4, five patients (36%) of the NM group had a score of less than 2 (no inflammation) compared with only one patient (11%) of the TC group. The inflammation score was not correlated to a difference in complaints. Only discomfort in patients with score 3 was lower ($p = 0.02$) compared to other groups (8.3 compared with 12.4, 14.4, and 13.8). After correction for additional medication, no decrease in inflammation was seen in any group.



Fig, 3. Adapted Merchant score (*p < 0.05, **p < 0.01).

Cavity infection

Before the study and during Visit 3, a bacterial swab was taken from all patients. In all patients, 27% of cavities were colonized by *Pseudomonas* species, 20% by *Staphylococcus aureus* (*S. aureus*), 30% with no or nonpathologic species, and the rest with other species (Table 3). Patients with *Pseudomonas aeruginosa* (*P. aeruginosa*), *S. aureus* and no or nonpathological colonization report the same VAS of discomfort (10.0, 13.4, 9.4), otorrhea (11.7, 12.6, 10.2), pain (4.6, 5.7, 2.6) and itching (7.8, 6.4, 7.1). Also, the same inflammation score of 2.8 was reported in all groups. After treatment, infection was reduced from 80% to 50% (eight to five patients) in the TC group and from 67% to 44% (12 to eight patients) in the NM group, as seen in Table 3.

Of four patients in the TC group who used additional medication, one patient with *P. aeruginosa* colonization used cotrimoxazole, one patients with *Pseudomonas fluorescens* used amoxicillin with clavulanic acid, one patient with *Proteus mirabilis* used ciprofloxacin and the fourth patient with a previous negative swab was treated with neomycine/polymyxine B eardrops and boronic acid powder. In the NM group, three patients received additional TC.

5

Table 3. Culture of microorganism per patient

Before Treatment (TC Group)	After Treatment (TC Group)	No. Patients
P. aeruginosa	*P. aeruginosa**	1
P. aeruginosa	*Aspergillus*	1
P. aeruginosa, BHSG	*P. aeruginosa*	1
P. aeruginosa	Excluded patient	1
P. mirabilis	*P. mirabilis* *	1
No path	*P. mirabilis, C.freundii**	1
No path	Excluded patient	1
S. aureus	No path	2
P. fluorescens	No path*	1
P. mirabilis	No path	1
No path	No path	1
		12
Before Treatment (NM Group)	**After Treatment (NM Group)**	**No. Patients**
P. aeruginosa	*P. aeruginosa*	2
P. aeruginosa, *S. aureus*	*P. aeruginosa*	1
S. aureus	*S. aureus*	2
S. aureus	*S. aureus* *	1
E. coli	*Candida**	1
Nonfermenter	*S. aureus* *	1
E. coli	No path	1
Klebsiella oxytoca	No path	1
Stenotrophomonas maltophilia	No path	1
Aspirgillus	No path	1
No path	No path	6
		18

The culture result is present for every patient at Visit 1 and Visit 3.
BHSG, beta-haemolytic *Streptococcus* group G; No path, no pathogenic species.
*Patient used additional medication.

Secondary outcome measures

No differences were observed between groups for total number of additional visits paid on Visit 3 and Visit 4 (p = 0.98 and 0.47, respectively) and for total use of additional medication (p = 0.60 and 0.52, respectively). Also, no difference was found for days until additional medication use (p = 0.70). As previously mentioned, four patients in the TC group and three patients in the NM group received additional medication on Visit 3. These patients had a total of five (TC group) and seven (NM group) additional visits during the whole study period. During and after Visit 3, two more patients in the TC group and 5 more patients in the NM group received additional medication and had one (TC group) and four (NM group) additional visits.

Adverse reaction

Seven patients in the NM group reported temporary irritation or feeling of pressure, and four patients reported short-term nausea or vertigo. In the TC group, irritation or feeling of pressure was reported 5 times and short-term nausea or vertigo two times. No serious adverse reactions with any correlation to medication use or study participation were reported.

Discussion

This study investigated the treatment effect of medical honey in comparison to conventional eardrops in chronically discharging open mastoid cavities. Topical treatment with honey resulted in less discomfort and otorrhea and an improvement of inflammatory status compared to eardrops. The treatment with honey did however result in an equal amount of extra medication need and revisits compared with eardrops.

A high number of patients with an open mastoid cavity suffer from therapy-resistant chronic discharge[18]. This causes social limitations and substantial health care costs by recurrent clinical visits, plethora of topical medications, and recurrent surgical interventions[7]. The cavity instability is suspected to be caused by primary tympanomastoid pathology, unfavorable cavity shape or size, and host factors[19]. Antibiotic/steroid eardrops have been proven as an effective treatment of acute otitis externa[20]. However, as shown in our study, many patients with an open mastoid cavity suffer from "resistant otorrhea", without improvement by chronic use of eardrops. Besides their desired effect, the use of antibiotics can results in bacterial resistance, which is seen in nearly half the patients with otitis. In infected open mastoid cavities an even higher incidence of resistance can be observed[21], with an increase after recurrent antibiotic treatment[22]. In addition, in more than 20% of patients with chronic ear disease[23], eardrops can lead to contact allergy, mostly caused by the components neomycin and framycetin[23,24], but also corticosteroids[25].

In our study, we considered honey as an alternative treatment for open cavity

5

problems because it is well known that honey serves as an effective alternative wound dressing[9], with only minor adverse events as local pain and irritation in wound treatment[11]. Honey has antimicrobial[26,27], antioxidative[28,29] and immune-modulatory capacities[30-32], without the disadvantaged of resistance formation.
Currently, we are testing the antioxidative capacity using Trolox Equivalent Antioxidative Capacity assay and electron spin resonance spectroscopy, which showed strong reactive oxygen species scavenging activity of NM.
Here, 27% of cavities were colonized with *Pseudomonas* species, 20% with *S. aureus* and 30% with no or nonpathologic species. Fungal species were only found in one patient. This is in line with other studies, in which chronic colonization was shown with similar species[33,34]. In this study, NM showed a bactericidal effect, similar to TC. Bacterial strains of *Escherichia coli* (*E. coli*) but not *S. aureus* or *P. aeruginosa,* were eradicated. Based on previous results from in vitro studies, we expected a broader effect of NM. Diluted NM source honey has a strong in vitro bactericidal activity against *E. coli*, *Pseudomonas* and against methicillin-resistant *S. aureus*[13,15], even in the form of biofilms[35]. This effect is amongst others attributed to methylglyoxal in NM[14], an important agent against bacterial biofilm[36]. It is possible that we identified not all bacteria that are responsible for cavity infection. Using normal swabs, only a few bacteria in biofilm can be detected[37], which are known to be responsible for a gross of chronic ear infection[38].
We could not find a relationship between complaints and colonization. So it seems that, in a patient with chronic cavity problems, a bacterial swab does not correlate to severity of complaints or bactericidal findings from in vitro studies.
A major finding in this study is that, after NM treatment, in 36% of patients, no inflammation was detectable anymore, compared with only 11% in the TC group. This means that the treatment with honey leads to complete objective absence of inflammation in a subgroup of patients.
So far, little is known about the histopathology of discharge from open cavities. It was shown previously that areas of the cavities are covered with ulceration and inflammatory granulation tissue adjacent to bone[5], with raised external canal humidity[39]. Mucosal factors that contribute to treatment failure still need to be investigated. In our study, we could only identify three patients with diabetes type 2 as an explicit host factor, which can influence the microenvironment and immune reaction in the cavity.
This is the first clinical study investigating an alternative add-on therapy in comparison to conventional eardrops for chronically discharging open mastoid cavities. In a relatively small group size, honey gel showed promising results.

Because of the decision to compare honey with eardrops, a blinding of patients' VAS was not possible.

In summary, we show for the first time that topical treatment with a medical honey gel is an alternative treatment option for patients who suffer from therapy-resistant chronically discharging open mastoid cavity. NasuMel led to a decrease in discomfort and otorrhea. Honey is a safe topical treatment agent without disadvantages of eardrops, such as bacterial resistance or contact allergy. However, more randomized studies with larger patient groups are needed to confirm our findings next to the investigation of the exact underlying mechanisms of this alternative treatment.

Conclusion

The topical use of medical honey is an alternative treatment for patients who suffer from therapy-resistant discharge from open mastoid cavity. Honey is a safe treatment agent without the unwanted disadvantages of eardrops and beneficial in reducing discomfort, otorrhea, and inflammation with a bactericidal effect.

References

1. Bercin S, Kutluhan A, Bozdemir Ket al. Results of revision mastoidectomy. *Acta Otolaryngol* 2009;129:138-41.
2. Mukherjee P, Saunders N, Liu Ret al. Long-term outcome of modified radical mastoidectomy. *J Laryngol Otol* 2004;118:612-6.
3. Minovi A, Venjacob J, Volkenstein Set al. Functional results after cholesteatoma surgery in an adult population using the retrograde mastoidectomy technique. *Eur Arch Otorhinolaryngol* 2013.
4. Aslan Felek S, Islam A, Celik Het al. The functional and anatomical results of the canal wall down tympanoplasty in extensive cholesteatoma. *Acta Otolaryngol* 2009;129:1388-94.
5. Youngs R. Temporal bone histopathology of open mastoidectomy cavities. *J Laryngol Otol* 1993;107:569-73.
6. Premachandra DJ, Woodward BM, Milton CMet al. Treatment of postoperative otorrhoea by grafting of mastoid cavities with cultured autologous epidermal cells. *Lancet* 1990;335:365-7.
7. Dornhoffer JL, Smith J, Richter Get al. Impact on quality of life after mastoid obliteration. *Laryngoscope* 2008;118:1427-32.
8. Schreml S, Szeimies RM, Prantl Let al. Wound healing in the 21st century. *J Am Acad Dermatol* 2010;63:866-81.
9. Molan PC. The evidence supporting the use of honey as a wound dressing. *Int J Low Extrem Wounds* 2006;5:40-54.
10. Molan PC. Potential of honey in the treatment of wounds and burns. *American journal of clinical dermatology* 2001;2:13-9.
11. Jull AB, Rodgers A, Walker N. Honey as a topical treatment for wounds. *Cochrane Database Syst Rev* 2008:CD005083.
12. Brolmann FE, Ubbink DT, Nelson EAet al. Evidence-based decisions for local and systemic wound care. *The British journal of surgery* 2012;99:1172-83.
13. Kwakman PH, de Boer L, Ruyter-Spira CPet al. Medical-grade honey enriched with antimicrobial peptides has enhanced activity against antibiotic-resistant pathogens. *Eur J Clin Microbiol Infect Dis* 2010.
14. Kwakman PH, te Velde AA, de Boer Let al. How honey kills bacteria. *Faseb J* 2010;24:2576-82.
15. Kwakman PH, Te Velde AA, de Boer Let al. Two major medicinal honeys have different mechanisms of bactericidal activity. *PLoS One* 2011;6:e17709.
16. Kwakman PH, Van den Akker JP, Guclu Aet al. Medical-grade honey kills antibiotic-resistant bacteria in vitro and eradicates skin colonization. *Clin Infect Dis* 2008;46:1677-82.
17. Merchant SN, Wang P, Jang CHet al. Efficacy of tympanomastoid surgery for control of infection in active chronic otitis media. *Laryngoscope* 1997;107:872-7.
18. Ishimoto S, Ito K, Sasaki Tet al. Total middle ear reconstructive surgery for the radicalized ear. *Otol Neurotol* 2002;23:262-6.

19. Gluth MB, Metrailer AM, Dornhoffer JLet al. Patterns of failure in canal wall down mastoidectomy cavity instability. *Otol Neurotol* 2012;33:998-1001.
20. Kaushik V, Malik T, Saeed SR. Interventions for acute otitis externa. *Cochrane Database Syst Rev* 2010:CD004740.
21. Saunders JE, Raju RP, Boone JLet al. Antibiotic resistance and otomycosis in the draining ear: culture results by diagnosis. *Am J Otolaryngol* 2011;32:470-6.
22. Saunders JE, Raju RP, Boone Jet al. Current bacteriology of suppurative otitis: resistant patterns and outcomes analysis. *Otol Neurotol* 2009;30:339-43.
23. Millard TP, Orton DI. Changing patterns of contact allergy in chronic inflammatory ear disease. *Contact dermatitis* 2004;50:83-6.
24. Holmes RC, Johns AN, Wilkinson JDet al. Medicament contact dermatitis in patients with chronic inflammatory ear disease. *J R Soc Med* 1982;75:27-30.
25. Devos SA, Mulder JJ, van der Valk PG. The relevance of positive patch test reactions in chronic otitis externa. *Contact dermatitis* 2000;42:354-5.
26. Basualdo C, Sgroy V, Finola MSet al. Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. *Veterinary microbiology* 2007;124:375-81.
27. Lusby PE, Coombes AL, Wilkinson JM. Bactericidal activity of different honeys against pathogenic bacteria. *Arch Med Res* 2005;36:464-7.
28. Alvarez-Suarez JM, Tulipani S, Diaz Det al. Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. *Food Chem Toxicol* 2010;48:2490-9.
29. Perez RA, Iglesias MT, Pueyo Eet al. Amino acid composition and antioxidant capacity of Spanish honeys. *J Agric Food Chem* 2007;55:360-5.
30. Al-Waili NS, Boni NS. Honey increased saliva, plasma, and urine content of total nitrite concentrations in normal individuals. *J Med Food* 2004;7:377-80.
31. Timm M, Bartelt S, Hansen EW. Immunomodulatory effects of honey cannot be distinguished from endotoxin. *Cytokine* 2008;42:113-20.
32. Tonks AJ, Cooper RA, Jones KPet al. Honey stimulates inflammatory cytokine production from monocytes. *Cytokine* 2003;21:242-7.
33. Gluth MB, Tan BY, Santa Maria PLet al. Unique microbiology of chronically unstable canal wall down tympanomastoid cavities: considerations for surgical revision. *J Laryngol Otol* 2013;127:458-62.
34. Pata YS, Ozturk C, Akbas Yet al. Microbiology of cerumen in patients with recurrent otitis externa and cases with open mastoidectomy cavities. *J Laryngol Otol* 2004;118:260-2.
35. Alandejani T, Marsan J, Ferris Wet al. Effectiveness of honey on Staphylococcus aureus and Pseudomonas aeruginosa biofilms. *Otolaryngol Head Neck Surg* 2009;141:114-8.
36. Kilty SJ, Duval M, Chan FTet al. Methylglyoxal: (active agent of manuka honey) in vitro activity against bacterial biofilms. *International forum of allergy & rhinology* 2011;1:348-50.
37. Daniel M, Imtiaz-Umer S, Fergie Net al. Bacterial involvement in otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2012;76:1416-22.

38. Fusconi M, Petrozza V, Taddei ARet al. Is biofilm the cause of chronic otitis externa? *Laryngoscope* 2011;121:2626-33.

39. Gray RF, Sharma A, Vowler SL. Relative humidity of the external auditory canal in normal and abnormal ears, and its pathogenic effect. *Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery* 2005;30:105-11.

CHAPTER 6

-

Treatment of recurrent eczematous external otitis with honey eardrops: a proof-of-concept study

Darius Henatsch, Cindy H. Nabuurs, Rens M. van de Goor, Petra F. Wolffs, Robert J. Stokroos

Otolaryngology - Head and Neck Surgery. 2017 Oct;157(4):696-699

Abstract

Eczematous external otitis is a chronic inflammatory disease and often difficult to treat. Our objective was to investigate the clinical effect and in vitro antibacterial potential of medical honey eardrops as treatment of eczematous external otitis. In a prospective study, 15 patients diagnosed with recurrent eczematous external otitis were treated with medical honey eardrops for 2 weeks. The following clinical outcomes were evaluated: visual analogue scale of ear complaints, score of eczema, and eradication of bacterial infection. Furthermore, the antibacterial effect of honey eardrops against different bacterial strains was tested in vitro. Treatment resulted in less discomfort and itching and decreased signs of eczema, with high patient satisfaction and without adverse reactions. Honey eardrops showed a strong in vitro inhibitory activity against all tested strains, but did not eradicate *Staphylococcus aureus* infection in vivo. The results of this preliminary study indicate a possible role of honey eardrops in eczematous ear disease.

Introduction

Eczematous external otitis is a chronic inflammatory condition, mainly characterized by pruritus, erythema, and scaling. Exacerbations can be accompanied by acute secondary bacterial infections[1]. Topical corticosteroids are the first choice for the treatment of this condition[2] and are mainly used during acute flares[3]. A combination with antibiotics is effective for acute suppurative otitis[4]. Nevertheless, the prolonged use of steroids and antibiotics has major disadvantages, such as skin atrophy[5], sensitization, and contact dermatitis[6,7]. The recurrent use of antibiotic eardrops can lead to bacterial resistance[8]. Honey is more often used as alternative treatment option for different diseases in otorhinolaryngology[9]. Honey has a strong antibacterial[10] and, to a lesser extent, antifungal property[11] and was found to be effective in some forms of dermatitis[12,13]. The effect of medical honey eardrops for external auditory canal disease and their bactericidal capacity has not been studied previously.

The aim of this study was to investigate the applicability and effect of medical honey eardrops as topical treatment of eczematous external otitis and to study their bactericidal capacity in vitro.

Methods

Patient selection

This study was performed at the Maastricht University Medical Center+ and approved by the ethical committee (protocol number 15-4-248). Patients with eczematous external otitis who were not treated 2 weeks prior to inclusion were recruited in a consecutive manner.

Intervention and outcome measures

After inclusion, patients completed a visual analogue scale (VAS; scale 0-10) about discomfort, otorrhea, pain, and itching of the affected ear. Standardized photos were taken of the external auditory canal using a rigid 0° otoscope, and a bacterial swab of the ear canal was taken. Patients were instructed to use honey eardrops (Otomel; Bfactory Health Products BV, Rhenen, the Netherlands) 3 times daily for 2 weeks. Patients returned after 1 week and again after 2 weeks. During both visits, a new VAS was completed; photos were taken; and during visit 3, a second bacterial swab was gathered. In addition, patients were asked to report treatment satisfaction. All standardized photos were scored by 2 blinded otologists on redness (score, 0-3: 0 = none, 3 = severe) and scaling (score, 0-3: 0 = none, 3 = severe) and scaling outside the ear canal (0 = none, 1 = introitus, 2 = concha).

Broth microdilution assay

The bactericidal activity of honey eardrops and acid eardrops with hydrocortisone 1% was compared and assessed against clinical and nonclinical isolates (in a 2-fold microbroth dilution assay). Strains were incubated overnight, and the minimal inhibitory dilution was determined as the first dilution at which no growth was visible.

Statistical analysis

Data was compared with the paired *t* test. Significance was determined to be at the confidence level of $P < .05$.

Results

Patient characteristics and clinical outcome

Fifteen patients were included in this study and treated with honey eardrops. Baseline characteristics are shown in Table 1.

Table 1. Baseline characteristics

Male:female, n	12:3
Age, y	63 (46-89)
Complaints, d	30 (7-360)
New patients, %	40
Treated earlier, %	87
Last visit outpatient clinics, d	58 (30-150)
Last treatment, d	60 (30-150)
Recurrent problem, %	87

Results are reported as median (range) unless noted otherwise.

Patients reported a baseline VAS score for discomfort of 5.3 ± 3.3 (mean ± SD), which was mainly based on itching (5.5 ± 3.8) and, to a lesser extend, otorrhea (1.8 ± 3.0) and pain (2.5 ± 3.2). After treatment, VAS scores for discomfort and itching decreased significantly to 3.6 ($P < .01$; 95% CI, 0.51-3.03) and 1.8 ($P < .01$; 95% CI, 1.58-5.89), respectively (Fig. 1).

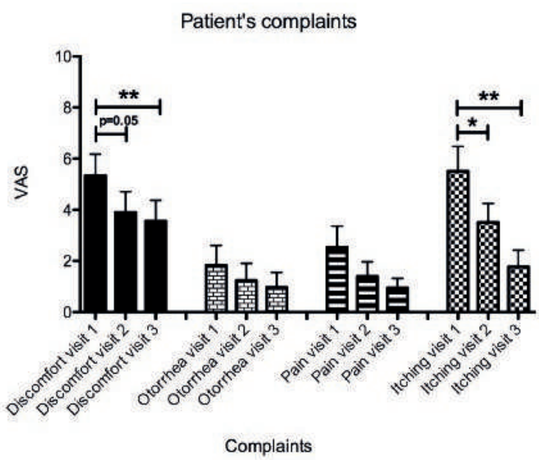


Fig. 1. Visual analog scale (VAS) for discomfort, otorrhea, pain, and itching at baseline (visit 1), after 1 week (visit 2), and after 2 weeks (visit 3) of treatment. Values are presented as mean ± SD. $^*P < .05$, $^{**}P < .01$)

6

Furthermore photos of the meatus, introitus, and concha were scored for redness and scaling before and after the treatment. The combined score decreased from 4.4 ± 1.7 before treatment to 3.1 ± 2.1 after it, which was statistically significant ($P = .02$, 95% CI, 0.15-2.28). Of all patients, a bacterial swab was taken before the treatment and at the end of the study. Four patients had an infection with *Staphylococcus aureus (S. aureus*) and 1 with *Candida parapsilosis*. The rest showed colonization with commensal bacteria. After treatment, the culture changed from commensal colonization to a negative culture in 4 patients (27%). *S. aureus* infections were not eradicated.

Patient satisfaction and adverse reaction

Patients rated the treatment on a nonvalidated 7-point scale (0 = very dissatisfied, 6 = extremely satisfied). Ten patients were very satisfied (score, 5); 4 patients, satisfied to moderately satisfied; and 1 patient, not satisfied (score, 2). No adverse reactions with any correlation to medication use or study participation were reported.

Antibacterial activity of honey eardrops

We tested the inhibitory activity of honey eardrops on different resistant and nonresistant pathogens, in comparison with acid eardrops with hydrocortisone 1%. Honey eardrops showed a high inhibitory activity, inhibiting bacteria at concentrations ranging from 3% to 12.5%, which was comparable to acid eardrops (Table 2) and independent of bacterial resistance.

Table 2. MIDs of honey eardrops vs acid eardrops with hydrocortisone 1%

MID		Otomel		Acid-drops		
ATCC number	**Strain**	1	2*	1	2*	
29213	*S. aureus*	1/8-1/16	1/8-1/16	1/8-1/16	1/8-1/16	
14990	*S. epidermidis*	1/8	1/8	1/8	1/16	
27853	*P. aeroginosa*	1/8	1/8	1/16	1/16	
25922	*E .coli*	1/8	1/8	1/16	1/16	
MID		**Otomel**		**Acid-drops**		**Strain type**
isolate	**strain**	1	2*	1	2*	
1	CNST	1/16	1/16	1/16	1/16	R
2	CNST	1/8	1/8	1/16	1/16	S
1	*E. coli*	1/4	1/4	1/16	1/16	R
2	E. coli	1/8	1/8	1/16	1/16	S
1	*P. aeroginosa*	1/8	1/8	1/16	1/16	S
2	*P. aeroginosa*	1/16	1/16	1/32	1/32	R
1	*S. aureus*	1/8	1/8	1/16	1/16	MRSA
2	*S. aureus*	1/16	1/16	1/16	1/16	R
3	*S. aureus*	1/8	1/8	1/16	1/16	S
1	*S. pneumoniae*	1/32	1/32	1/32	1/32	S
2	*S. pneumoniae*	1/32	1/32	1/64	1/64	R

Abbreviations: ATCC, American Type Culture Collection; CNST, Coagulase-negative staphylococci; E. coli, *Escherichia coli*; MRSA, methicillin-resistant *S. aureus*; MID, minimum inhibitory dilution; *P. aeroginosa*, *Pseudomonas aeruginosa*; R, resistant; S, susceptible; *S. aureus, Staphylococcus aureus*; S. pneumoniae, *Streptococcus pneumoniae.* *Duplicate

Discussion and conclusion

The pathophysiology of eczema involves a malfunction of the skin barrier[14]. Disadvantage of common topical preparations are skin atrophy[5], sensitization[6,7,] and bacterial resistance[8]. Honey mixture was an effective treatment in different types of dermatitis[12,13,15] and effective in animal external otitis and pruritus, without undesirable side effects[16].

In our study, honey eardrops reduced discomfort and mainly pruritus. We found a high in vitro inhibitory activity of honey droplets, with strong efficiency against all strains. However, honey treatment could not eradicate *S. aureus* infections in vivo and led only to less commensal colonization. The poor eradication of *S. aureus* is in line with other studies[17], possibly by growth within hair follicles[18] and redistribution from deeper skin layers[19]. This study was designed as a proof-of-concept study, with limitations including a small study size and the lack of a control group.

We showed that honey eardrops have a possible role in the treatment of eczematous ear disease, by reducing complaints and clinical signs of eczema and by a strong in vitro inhibitory activity against bacteria. This clears the way for further randomized studies.

References

1. Celebi Erdivanli O, Kazikdas KC, Ozergin Coskun Z, et al. Skin prick test reactivity in patients with chronic eczematous external otitis. Clin Exp Otorhinolaryngol 2011;4:174-6.
2. Jacobsson S, Karlsson G, Rigner P, et al. Clinical efficacy of budesonide in the treatment of eczematous external otitis. Eur Arch Otorhinolaryngol 1991;248:246-9.
3. Eichenfield LF, Tom WL, Berger TG, et al. Guidelines of care for the management of atopic dermatitis: section 2. Management and treatment of atopic dermatitis with topical therapies. J Am Acad Dermatol 2014;71:116-32.
4. Kaushik V, Malik T, Saeed SR. Interventions for acute otitis externa. Cochrane Database Syst Rev 2010:CD004740.
5. Lubach D, Bensmann A, Bornemann U. Steroid-induced dermal atrophy. Investigations on discontinuous application. Dermatologica 1989;179:67-72.
6. Sood S, Strachan DR, Tsikoudas A, et al. Allergic otitis externa. Clin Otolaryngol Allied Sci 2002;27:233-6.
7. Fraki JE, Kalimo K, Tuohimaa P, et al. Contact allergy to various components of topical preparations for treatment of external otitis. Acta Otolaryngol 1985;100:414-8.
8. Saunders JE, Raju RP, Boone JL, et al. Antibiotic resistance and otomycosis in the draining ear: culture results by diagnosis. Am J Otolaryngol 2011;32:470-6.
9. Henatsch D, Wesseling F, Kross KW, et al. Honey and beehive products in otorhinolaryngology: a narrative review. Clin Otolaryngol 2016;41:519-31.
10. Lu J, Carter DA, Turnbull L, et al. The effect of New Zealand kanuka, manuka and clover honeys on bacterial growth dynamics and cellular morphology varies according to the species. PLoS One 2013;8:e55898.
11. Estevinho ML, Afonso SE, Feas X. Antifungal effect of lavender honey against Candida albicans , Candida krusei and Cryptococcus neoformans. J Food Sci Technol 2011;48:640-3.
12. Al-Waili NS. Clinical and mycological benefits of topical application of honey, olive oil and beeswax in diaper dermatitis. Clin Microbiol Infect 2005;11:160-3.
13. Al-Waili NS. Therapeutic and prophylactic effects of crude honey on chronic seborrheic dermatitis and dandruff. Eur J Med Res 2001;6:306-8.
14. Bos JD, Brenninkmeijer EE, Schram ME, et al. Atopic eczema or atopiform dermatitis. Exp Dermatol 2010;19:325-31.
15. Al-Waili NS. Topical application of natural honey, beeswax and olive oil mixture for atopic dermatitis or psoriasis: partially controlled, single-blinded study. Complement Ther Med 2003;11:226-34.
16. Maruhashi E, Braz BS, Nunes T, et al. Efficacy of medical grade honey in the management of canine otitis externa - a pilot study. Vet Dermatol 2016;27:93-8e27.
17. Birnie AJ, Bath-Hextall FJ, Ravenscroft JC, et al. Interventions to reduce Staphylococcus aureus in the management of atopic eczema. Cochrane Database Syst Rev 2008:CD003871.

18. Ten Broeke-Smits NJ, Kummer JA, Bleys RL, et al. Hair follicles as a niche of Staphylococcus aureus in the nose; is a more effective decolonisation strategy needed? J Hosp Infect 2010;76:211-4.
19. Onunkwo CC, Hahn BL, Sohnle PG. Clearance of experimental cutaneous Staphylococcus aureus infections in mice. Arch Dermatol Res 2010;302:375-82.

CHAPTER 7

-

Summary and general discussion

The objectives of this thesis were to investigate the clinical applicability of medical grade honey in 2 different chronic ear conditions and to explore the underlying mechanism of the wound healing effect of honey. Prior to the planning and conduction of our clinical work, no studies existed, which described medical honey as a topical treatment in conditions of the external ear canal and its vicinity. We started our work with a literature review, to summarize the therapeutic efficacy and applicability of honey and other beehive products in otorhinolaryngology. By systematically evaluating 36 human studies, we showed that honey could play a role in the treatment of mucositis, childhood cough, persistent post-infectious cough, post-tonsillectomy wound healing and the prevention of otitis media. In addition, propolis was shown to be beneficial in the treatment of (aphtous) stomatitis and mouth ulcers, and royal jelly reduced mucositis.
Inspired by this therapeutic potential of honey, we performed 2 clinical studies to investigate the applicability and potential underlying mechanisms of medical honey in patients suffering from eczematous external otitis or a discharging open mastoid cavity. This research was accompanied by a histopathology study, focusing on the origin of chronically discharging cavities. In vitro studies were performed as well to investigate the underlying antibacterial and antioxidative potential of honey to get more insight into the potential underlying mechanisms of the wound healing capacity of medicinal honey. In the following paragraphs obtained results are summarized and put into perspective.

Wound healing potential of medical honey

The wound healing potential of medical honey is based on antiseptic, antioxidative, immunemodulatory and wound cleaning properties. In **chapter 3 and 6** the antibacterial activity of *Revamil* (the source honey of the honey eardrops *Otomel* and the honey gel *Nasumel*) and honey eardrops was examined. At 12.5%, the source honey inhibited the growth of *E. coli*, *S. aureus* and *P. aeroginosa*. Honey eardrops had an inhibitory activity on different resistant and nonresistant pathogens at concentrations ranging from 3% to 12.5%. In both clinical studies (**chapter 5 and 6**) we showed that in eczematous external otitis the pathogens *S. aureus* and in open mastoid cavities the pathogens *P. aeroginosa* and *S. aureus* predominate. Interestingly, in both clinical studies the in vivo eradication of especially *S. aureus*, but also *P. aeroginosa* was less effective, as expected from in vitro outcomes. It is known that antibacterial properties of the source honey of honey eardrops and gel is based on a high accumulation of hydrogen peroxide upon dilution, the cationic compound bee defensin-1 and a low pH of about 3.5[1]. In **chapter 3**, we demonstrated

that this source honey contains the α-oxaldehydes MG, GO and 3-DG and free amino acids. In our experiments, MG and GO both formed different (carbon centered) radicals in reaction with amino acids and/or hydrogen peroxide, which are both present in this honey. This proves that besides an important peroxide dependent antiseptic effect, also a mixed and non-peroxide antiseptic effect is present in this type of honey. In addition, we showed that non-peroxide (Manuka) honeys have much higher concentrations of MG, which seems to be the most important antibacterial factor in this kind of honey by forming different radicals.
In the in vitro work it was shown that one of the most important characteristics of honey is based on the formation of reactive oxygen radicals. Whereas another important property of honey is the quenching of radicals, especially important in chronic wounds[2]. All medical honeys had a strong antioxidative capacity against the highly reactive hydroxyl and superoxide radicals, both present during inflammation in wounds[2].

Applicability of medical honey in chronic ear conditions

In 2 clinical studies, we investigated the applicability of medical honey in patients with chronic ear conditions. In the first study (**chapter 5**), the treatment of patients with a chronically infected open mastoid cavity with medical honey gel led to less discomfort, otorrhea and inflammation and reduced bacterial infection. In this study honey was compared to conventional eardrops and offered a fair treatment alternative. It was shown in a histopathology study in **chapter 4** that the treatment success of both treatments was dependent on sort of tissue coverage of the cavity. Cavities were covered either with stratified squamous (keratinized) epithelium, respiratory columnar epithelium or granulation tissue. When covered with respiratory epithelium healing was shown to be less likely.
In the second clinical study (**chapter 6**), patients with eczematous external otitis were treated with medical honey eardrops. This led to a reduction of discomfort, itching and signs of eczema and a high patient satisfaction.
In otorhinolaryngology, honey is mainly used to treat (chronic) mucosal inflammatory conditions, as shown in **chapter 2**. In conditions of the oral mucosa, honey positively influenced onset, healing-time and pain, with a reduction of candida and bacterial infection. In addition, honey had a positive effect during acute inflammation of pharyngeal and laryngeal mucosa. In **chapter 4** it was presented that in all tissue biopsies of open mastoid cavities inflammatory infiltrations were present, consisting of amongst others lymphocytes and plasma cells. An important finding of this study is that lesions in progression towards a 'mucosa-

like' (respiratory (ciliated) columnar) epithelium are more susceptible to infection and less prone to healing. In all these acute or chronic inflammatory (mucosal) conditions, oxidative stress is an important factor during healing and progression to chronicity[2,3]. Also in the pathogenesis of eczematous disease, oxidative stress seems to be a key factor[4]. We proved in **chapter 3** that medical honey is an appropriate scavenger of different reactive oxygen radicals, which perpetuate non-healing wounds[3]. This effect, together with the known immunemodulatory potential of medical honey can explain the therapeutic potential, as observed in both clinical studies in **chapter 5 and 6**.
Besides these active wound healing potential, medical honey has another, quite important quality: the absence of undesirable adverse events. 'Common' therapies in otology have several unwanted side effects. The application of corticosteroids can cause permanent skin atrophy[5] and other skin reactions[6]. In addition, various components of topical preparations lead to sensitization[7,8] and contact allergy[9] and the recurrent use of antibiotic eardrops leads to bacterial resistance[10,11].
In the narrative review (**chapter 2)** and both clinical studies (**chapter 5 and 6)**, only mild adverse reactions in a low percentage of patients were reported, which only consisted of mild itching (when applied locally).

Conclusion and future perspective

In summary, in this thesis we showed that medical honey can play an important role in the treatment of the chronic ear conditions discharging open mastoid cavity and eczematous external otitis. In addition we proved that the wound healing capacity of medical honey is based on a strong antiseptic capacity, partly due to the interaction of α -oxaldehydes, hydrogen peroxide and amino acids, and scavenging capacity of free oxygen radicals. Importantly, the wound healing characteristics of medical honey were not accompanied by undesired side effect.
Nowadays, medical professional face the problem of a missing diversity and the lack of extension of pharmaceuticals. Unless revolutionary progression in biomedical science, most otolaryngologists still use boric acid powder and gentian violet for the treatment of discharging open mastoid cavities, both remedies that are already used for decades. Nowadays, the developmental path of a medical product is increasingly expensive, challenging and inefficient, which leads to a decline in new drug registration and an increase in product costs[12]. Especially in the development of new antibiotic agents the pharmaceutical industry has faced economic and regulatory obstacles[13].
In this thesis it was shown that 'rediscovering' a known remedy can contribute

to the solution of this problem. The modification of medical (source) honey to droplets or gel can further extent the area of application in otorhinolaryngology. Importantly, the differences of the floral source of honeys lead to significant differences in the phytochemical profile of honeys[14]. For further development and comparison of studies, standardization is unavoidable. At the moment not only 2 different families of honey, but also numberless unique honeys are used in in vitro work and clinical studies.

Based on this broad diversity of natural honeys with all unique compositions, it is impossible to advise a single honey product. Restrictions of this diversity and good transnational agreements by special task forces could enhance and broaden the future use of medical honey.

References

1. Kwakman PH, te Velde AA, de Boer L, Speijer D, Vandenbroucke-Grauls CM, Zaat SA. How honey kills bacteria. Faseb J 2010; 24:2576-2582.
2. Schafer M, Werner S. Oxidative stress in normal and impaired wound repair. Pharmacol Res 2008; 58:165-171.
3. Salim AS. The role of oxygen-derived free radicals in the management of venous (varicose) ulceration: a new approach. World J Surg 1991; 15:264-269.
4. Ji H, Li XK. Oxidative Stress in Atopic Dermatitis. Oxidative medicine and cellular longevity 2016; 2016:2721469.
5. Lubach D, Bensmann A, Bornemann U. Steroid-induced dermal atrophy. Investigations on discontinuous application. Dermatologica 1989; 179:67-72.
6. Cury Martins J, Martins C, Aoki V, Gois AF, Ishii HA, da Silva EM. Topical tacrolimus for atopic dermatitis. Cochrane Database Syst Rev 2015:CD009864.
7. Sood S, Strachan DR, Tsikoudas A, Stables GI. Allergic otitis externa. Clinical otolaryngology and allied sciences 2002; 27:233-236.
8. Fraki JE, Kalimo K, Tuohimaa P, Aantaa E. Contact allergy to various components of topical preparations for treatment of external otitis. Acta Otolaryngol 1985; 100:414-418.
9. Millard TP, Orton DI. Changing patterns of contact allergy in chronic inflammatory ear disease. Contact dermatitis 2004; 50:83-86.
10. Saunders JE, Raju RP, Boone JL, Hales NW, Berryhill WE. Antibiotic resistance and otomycosis in the draining ear: culture results by diagnosis. Am J Otolaryngol 2011; 32:470-476.
11. Saunders JE, Raju RP, Boone J, Berryhill W. Current bacteriology of suppurative otitis: resistant patterns and outcomes analysis. Otol Neurotol 2009; 30:339-343.
12. FDA. Innovation or stagnation: challenges and opportunity on the critical path to new medical products, 2004.
13. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T 2015; 40:277-283.
14. Shah A, Amini-Nik S. The Role of Phytochemicals in the Inflammatory Phase of Wound Healing. Int J Mol Sci 2017; 18.

Samenvatting (Dutch)

Twee chronische aandoeningen van het oor zijn enerzijds eczeem van de gehoorgang en anderzijds de chronisch ontstoken radicaalholte. Beide aandoeningen kunnen moeilijk behandelbaar zijn en een chronisch en recidiverend beloop hebben. De veelal standaard toegepaste behandeling tijdens een actieve ontstekingsfase bestaat uit het toedienen van oordruppels met corticosteroïden met of zonder antibiotica. Helaas brengt deze vorm van behandeling vaak nadelige gevolgen met zich mee, zoals contactallergie en de vorming van resistente bacteriën in het oor.
Medische honing staat van oudsher bekend als middel voor wondgenezing met weinig bijwerkingen en zou een alternatieve behandeloptie kunnen bieden.
Dit proefschrift beschrijft de resultaten van een literatuur studie, twee klinische studies, een histopathologie studie en laboratorium onderzoeken, met als doel de therapeutische toepasbaarheid van medische honing bij de behandeling van chronische oorontstekingen te onderzoeken.

Binnen de KNO heelkunde wordt (medische) honing frequent als behandelingsmethode van een brede scala van aandoeningen toegepast. In de literatuur studie (**hoofdstuk 2**) laten 36 studies zien, dat honing een genezende werking heeft op mucositis, bij hoesten van kinderen en hoesten als gevolg van een bovenste luchtweginfectie, dat het tevens de wond genezing na een keelamandeloperatie bevorderd en dat het ontstaan van een middenoor ontsteking hiermee voorkomen kan worden. Verder bleek dat andere producten van de honingbij, zoals propolis en royal jelly, ook een genezende werking lijken te hebben bij met name mondaften en mucositis.
Dit proefschrift beschrijft verder 2 klinische studies. Patiënten gediagnosticeerd met een chronisch ontstoken radicaalholte, werden behandeld met een honinggel (*Nasumel*) (**hoofdstuk 5**) en patiënten gediagnosticeerd met chronisch ooreczeem werden behandeld met honing oordruppels (*Otomel*) **(hoofdstuk 6)**. In de gerandomiseerde klinische studie in **hoofdstuk 5** werden patiënten met een chronisch ontstoken radicaalholte 2 keer binnen 8 weken met honinggel behandeld. Deze behandeling werd vergeleken met conventionele oordruppels. De honingbehandeling resulteerde in minder ontsteking, minder ongemak en tevens minder looporen. Ook de aantal actieve infecties daalde. Deze klinische studie werd aangevuld door een histopathologische studie (**hoofdstuk 4**) om inzicht te krijgen in de onderliggende pathologische mechanismen van een ontstoken holte.

7

Resultaten lieten zien dat een ontstoken holte uit 3 verschillen soorten bekleding bestaat. De bekleding bestond deels uit meerlagig, afgeplat verhoornd epitheel, cilindrisch, trilhaar dragend epitheel of granulatie weefsel zonder epitheliale bekleding. De behandeling sloeg het minste aan bij patiënten met cilindrisch epitheel in de radicaalholte, waarschijnlijk door de samenstelling van immuun cellen in het onderliggende weefsel en een verhoogde vatbaarheid voor infecties. Een biopsie van een ontstoken holte kan dus een toegevoegde waarde bieden in de keuze van de behandelstrategie.
In de tweede klinische studie welke gepresenteerd wordt in **hoofdstuk 6**, werden patiënten met chronisch ooreczeem behandeld met honing oordruppels. Deze patiënten waren zeer tevreden met de behandeling en ongemak van het oor en klachten van jeuk namen na 2 weken af. Bij patiënten met een chronisch ooreczeem werd vaak een infectie met *S. aureus* aangetroffen, terwijl bij patiënten met een ontstoken radicaalholte vaak de *S. aureus* of *P. aeroginosa* uit de kweek aangetoond werden.
De (oppervlakkige) behandeling met medische honing bleek in beide klinische studies als zowel voortkomend uit de literatuurstudie, enkel in sporadische gevallen een zeer milde lokale irritatie als bijwerking te hebben.
Naast de klinische werking werd de potentiële mechanistische werking van medische honing onderzocht. De antibacteriële werking van verschillen soorten medische honing werd getest, waaronder de honing *Revamil* (basishoning van het honinggel *Nasumel* en de honing oordruppels *Otomel*) en de honing oordruppels. Revamil remde de groei van de bacteriën *Escherichia coli*, *S. aureus* en *P. aeroginosa* bij 12,5% volledig. Honing oordruppels met concentraties van 3% tot 12,5% lieten deze werking tevens bij vele andere (resistente) soorten bacteriën zien.
Verder werd er onderzoek uitgevoerd om het onderliggende mechanisme van deze antibacteriële werking in kaart te brengen (**hoofdstuk 3).** Er werd aangetoond, dat 2 soorten medische honing, namelijk honing met en zonder peroxide werking, verschillende concentraties van de α-oxaldehyden methylglyoxal (MG), glyoxal (GO) en 3-deoxyglucoson (3-DG) bevatten, alsook verschillende hoeveelheden vrije aminozuren. MG en GO kunnen makkelijk vrije radicalen vormen door reactie aan te gaan met waterstof peroxide en/of de aminozuren lysine en arginine. Resultaten lieten zien dat MG en GO een zeer sterk remmende werking hebben op de groei van bacteriën. **Hoofdstuk 3** toont tevens de belangrijke bevinding dat beide soorten honing naast de MG en GO geïnduceerde, radicalen-afhankelijke antibacteriële werking, juist ook zuurstofradicalen kunnen wegvangen. Wij lieten zien dat de medische honing werkt als een sterke ‘scavenger’ van de hydroxyl- en superoxide

radicalen. Deze beide soorten radicalen komen veel in chronische wonden voor, zorgen voor oxidatieve stress en een vertraging van de wondgenezing.
Kortom, dit proefschrift laat zien dat medische honing een potentiele rol kan spelen bij de behandeling van chronisch ooreczeem en de chronisch ontstoken radicaalholte. De onderliggende wond genezende eigenschappen komen door een antibacteriële werking, deels gebaseerd op radicaalvorming met α-oxaldehyden, maar juist ook het wegvangen van zuurstofradicalen.

Zusammenfassung (German)

In der Otologie sind mehrere Krankheitsbilder bekannt, die einen chronischen oder chronisch-rezidivierenden Charakter haben. Dazu gehören das chronische Gehörgangekzem und die chronisch entzündete offene Mastoidhöhle. Das Schwierige bei diesen otologischen Erkrankungen ist die oft andauernde und erfolgslose Behandlung mit verschiedenen lokalen Medikationen.

Honig mit seiner einzigartigen Zusammensetzung ist das älteste bekannte Wundheilmittel, und könnte eine gute alternative Behandlung darstellen.

In der vorliegenden Promotionsschrift werden die Resultate von einer Literaturstudie, zwei klinischen Studien, einer histopathologischen Studie und experimentellen Untersuchungen präsentiert und evaluiert, mit dem gemeinsamen Ziel, die therapeutische Wirkung von medizinischem Honig bei der Behandlung von chronischen Ohrinfektionen zu erforschen.

Am Anfang der Arbeit zeigen wir anhand einer Literaturzusammenfassung (**Kapitel 2**) von 36 humanen Studien, dass medizinischer Honig und die der Biene entstammenden Produkte Propolis und Royal Jelly, bei mehreren Erkrankungen im HNO Bereich bereits angewendet wurden. Honig erzeugte eine heilende Wirkung bei Mukositis, kindlichem Husten, Husten nach Rachenentzündung, sowie nach Rachenmandeloperationen und kann das Auftreten von Mittelohrentzündungen reduzieren. Propolis und Royal Jelly zeigten eine therapeutische Wirkung bei Aphten und Mucositis.

Basierend auf diesen Ergebnissen wurde die Wirkung von medizinischem Honig-Gel bei Patienten mit einer chronisch entzündeten offenen Mastoidhöhle getestet. **Kapitel 5** beschreibt die Resultate einer prospektiven, randomisierten Studie, in der Patienten mit Honig-Gel oder konventionellen Lokaltherapeutika (Antibiotika/ Kortikosteroid Ohrtropfen) behandelt wurden. Nach der Behandlung mit Honig hatten Patienten weniger Beschwerden und Otorrhoe, ebenso eine Verminderung der Entzündung und der Infektionen ihrer offenen Mastoidhöhle. Diese klinische Studie wurde ergänzt durch eine histopathologische Studie (**Kapitel 4**). Aufgrund von Biopsien von entzündeten offenen Mastoidhöhlen, wurde festgestellt, dass die Auskleidung einer solchen Höhle (partiell) aus 3 verschiedenen Typen bestehen kann, nämlich verhornendem Plattenepithel, kubischem (Flimmer-) Epithel oder Granulationsgewebe ohne Epithelbekleidung. Interessanterweise konnten wir hierbei nachweisen, dass eine (partielle) Bekleidung mit kubischem (Flimmer-) Epithel die Prognose einer Genesung verschlechtert, unabhängig von der Behandlungsstrategie. Wahrscheinlich sind hierfür eine höhere

Infektionsanfälligkeit dieses Gewebes und Unterschiede in der Zusammensetzung von Immunzellen in tiefergelegenen Gewebsschichten verantwortlich.
In einer weiteren klinische Studie (**Kapitel 6**) wurden Patienten mit chronischem Ohrekzem über 2 Wochen mit Honig-Ohrentropfen behandelt. Die Patienten waren mit der Therapie sehr zufrieden, subjektive Beschwerden wie Juckreiz nahmen ab. Als Mikroorganismus wurde bei Patienten mit Ohrekzem hauptsächlich *Staphylococcus aureus* angetroffen, wohingegen die meisten Mastoidhöhlen mit *Pseudomonas* oder *Staphylococcus aureus* infiziert waren.
Neben den klinischen Studien wurden auch experimentelle Untersuchungen und Aktivitätsbestimmungen von verschiedenen Honigsorten gegen ein breites Mikroorganismenspektrum durchgeführt. In **Kapitel 3 und 6** wird erläutert, dass der Honig *Revamil* (Basishonig für das Honig-Gel und die Honig-Ohrentropfen) eine antiseptische Wirkung bei einer Konzentration von 12,5% erreicht, und das Wachstum der Stämme *Escherichia coli*, *Staphylococcus aureus* und *Pseudomonas aeroginosa* vollständig hemmt. Honig-Ohrentropfen erreichten eine vergleichbare Wirkung gegen ein breites (resistentes) Mikroorganismenspektrum bei Konzentrationen von 3% bis 12.5%.
In **Kapitel 3** wurde der Frage nachgegangen, wie ein Teil dieser antiseptischen Wirkung zu erklären ist. Wir konnten bestimmen, dass Honigsorten der Peroxide- und Nicht-Peroxide-Gruppe verschiedene Konzentrationen der α-Oxaldehyde Methylglyoxal (MG), Glyoxal (GO) und 3-Deoxyglucoson beinhalten können, sowie unterschiedliche Mengen von freien Aminosäuren. MG und GO können mehrere Radikale mit Wasserstoffperoxid und/oder den Aminosäuren Lysin und Arginin bilden. Des Weiteren haben diese beiden α-Oxaldehyde eine sehr starke antiseptische Eigenschaft. Neben der teilweise auf Radikalbildung basierenden antiseptischen Wirkung, konnten wir interessanter Weise auch eine antioxidative Wirkung, also eine Radikalfängerfunktion, bei allen medizinische Honigsorten feststellen. Diese Eigenschaft wurde an Hydroxyl- und Superoxid-Radikalen getestet. Beide Radikale spielen eine wichtige Rolle bei der Persistenz von chronischen Wunden durch oxidativen Stress.

Zusammenfassend wurde in dieser Promotionsschrift erwiesen, dass medizinischer Honig einen wertvollen Beitrag bei der Behandlung des chronischen Ohrekzems und der chronisch entzündeten offenen Mastoidhöhle leisten kann. Ein Teil der therapeutischen Eigenschaft von medizinischem Honig basiert auf der Wechselwirkung von antiseptischen Eigenschaften durch die Bildung von Radikalen und der Verminderung von oxidativen Stress durch das Einfangen von Radikalen.

Valorisation

Relevance

Most physicians have to deal with the prevention and treatment of infections and inflammations. The annual incidence of infection of the outer ear canal (external otitis) is about 1% of the population of the general practitioner[1] and up to 10% at the otolaryngologist[2]. Chronic ear infection, due to an underlying condition, as eczema or after ear operations, have a high socioeconomic impact and cause significant health care costs[3]. In this patient group, the chronic or recurrent treatment with antibiotics causes an additional problem, which is bacterial resistance. The increase of bacterial resistance, together with a decrease in the development of new antibiotic agents, illustrated the need for new antiseptic strategies, as mentioned earlier.

This thesis focuses on the use of the antiseptic agent honey, and it's clinical relevance for patients with chronic ear infections. The refinement of medical honey for clinical use does not only offer a new treatment for patients with chronic ear infections, but additionally contributes to the important development of new alternatives in the fight against bacterial resistance.

The results of this thesis offer an overview of the clinical applicability of medical honey in otorhinolaryngology, provide insights in properties and working mechanisms of different sorts of honey, and prove safety and effectiveness of honey treatment in patients with chronic ear infections.

Target population

The results of this thesis are relevant for health care professionals, patients, as well as companies interested in drug research and development. Both clinical, and the histopathology study show for the first time, that honey can be used as a treatment in chronic ear infections. This is very important for care professionals and patients. For patients with chronic ear eczema or a chronically infected open mastoid cavity, this treatment offers a new safe strategy, which can be used after the failure of other therapies or as an additional treatment without disadvantages known from 'conventional' treatments. Health care professionals are offered a whole new antiseptic strategy. Medical honey and honey droplets are available to buy and ready to use.

In addition, the results of this thesis can be important for companies (profit and non-profit), who are involved in the development and refinement of antiseptic agents. We provide additional information on possible working mechanisms of different sorts of honey, but also present an overview of a broad applicability (in our review) and show the additional option of safe use in ear infection in the form of a gel and droplets.

Products

In this thesis we focused on three different products. Medical honey in its source form, honey gel, and honey droplets. To understand underlying working mechanisms, it was chosen to investigate the antiseptic effects and antioxidative potential of peroxide (Revamil) and non-peroxide (Manuka) medical source honeys. For clinical use honey was also tested in droplet (Otomel) and gel (Nasumel) form.

Innovation, realisation and costs

In the systematic review, which is provided in this thesis, it was shown that there is no scientific literature about the use of medical honey as a topical treatment in ear infection. The clinical studies, which are presented in this thesis, are the first showing usefulness and safety of honey in these conditions. All products are already available, ready to use, with a simple applicability. We proved that honey droplets and honey gel are safe, with only minor adverse events. The costs of both, honey eardrops and honey gel, are low and comparable to common eardrops used for ear infections.

References

1. Okkes IM OS, Lamberts H. Van klacht naar diagnose: episodegegevens uit de huisartspraktijk. Bussum: Coutinho, 1998.
2. Bojrab DI, Bruderly T, Abdulrazzak Y. Otitis externa. Otolaryngol Clin North Am 1996; 29:761-782.
3. Dornhoffer JL, Smith J, Richter G, Boeckmann J. Impact on quality of life after mastoid obliteration. Laryngoscope 2008; 118:1427-1432.

Acknowledgements

During the last years many people have contributed to the work, which forms the basis of this thesis and/or supported me during this process.

I would like to thank especially the following people for their support:

All the members of the manuscript committee: Prof. Kremer, Prof. Heeneman, Prof. Steijlen, Prof. Coert and Dr. Pennings.

My promotor Prof. Robert Stokroos: Dear Robert, because of your support I started and finished this research project and my residency. Thank you very much for your help during the last years.

My copromotor Dr. Jacco Briedé: Dear Jacco, you always helped me unconditionally and motivated me very much. You truly are a top academic mentor.

All colleges of the department Otorhinolaryngology MUMC+ for their help during the clinical studies, especially Prof. Bernd Kremer for being such an encouraging and exemplary head of the department.

All co-authors, who helped me to perform the clinical studies and the laboratory work.

In particular, I thank Esther for being my partner, for all the mental support, scientific knowledge and hours you had to listen to 'honey' and 'ears'.

My parents, who always stimulated and supported my academic career.

Curriculum vitae

Darius Henatsch was born in Cologne, Germany, in 1984. After finishing secondary school in Cologne he moved to Maastricht in 2004 to study *Molecular Life Science* at Maastricht University. After receiving the Bachelor of Science in 2007 he continued with the Master studies *Physician – Clinical Investigator* and obtained his Medical Doctor and Master of Science degree in 2011. Subsequently, Darius started the residency Otolaryngology at the Maastricht University Medical Center+, under supervision of Prof. Dr. Bernd Kremer and Prof. Dr. Robert Stokroos. In his last year, Darius differentiated in head and neck surgery at MUMC+ and spend three months in Germany for differentiation in reconstructive surgery (University Hospital Bonn) and head and neck and laser surgery (University Hospital Schleswig- Holstein, Campus Kiel). After finishing the residency in Sep 2016, the Fellowship 'Facial plastic and reconstructive surgery' (Oct 2016 – Sep 2017) was completed in Gelderse Vallei Hospital (Ede) under supervision of Dr. Wilbert Boek and Radboud University Medical Center (Nijmegen), under supervision of Dr. Koen Ingels and Dr. Niels van Heerbeek.

Darius worked one year as Otolaryngologist in Zuyderland Medical Center (Heerlen/ Sittard) from Oct 2017 – Sep 2018. From Oct 2018 he is working at Viecuri Medical Center (Venlo) and is performing reconstructive operations at Mauritskliniek (Den Haag).

He is living in partnership with Esther Phielix in Maastricht. Together they have three children, Julius, Lennart and Sophie.

List of publications

Henatsch D, den Hartog GJ, Duijvestijn AM, Wolffs PF, Phielix E, Stokroos RJ, Briedé JJ: The contribution of α-dicarbonyl compound dependent radical formation to the antiseptic effect of honey. J Funct Foods. 2018 Jun;45(6):239-246.

Henatsch D, Alsulami S, Peutz-Kootstra CJ, Duijvestijn AM, Cleutjens JP, Stokroos RJ: Histopathology and inflammatory features of chronically discharging open mastoid cavities. JAMA Otolaryngol Head Neck Surg. 2018 Mar 1;144(3):211-217.

Henatsch D, Nabuurs CH, van de Goor RM, Wolffs PF, Stokroos RJ: Treatment of recurrent eczematous external otitis with honey eardrops: a proof-of-concept study. Otolaryngol Head Neck Surg. 2017 Oct;157(4):696-699.

Henatsch D, Wesseling F, Kross KW, Stokroos RJ: Honey and beehive products in otorhinolaryngology: a narrative review. Clin Otolaryngol. 2016 Oct;41(5):519-531.

Henatsch D, Wesseling F, Briedé JJ, Stokroos RJ: Treatment of chronically infected open mastoid cavities with medical honey: a randomized controlled trial. Otol Neurotol. 2015 Jun;36(5):782-787.

van Leeuwen M, Kemna MJ, de Winther MP, Boon L, Duijvestijn AM, **Henatsch D**, Bos NA, Gijbels MJ, Tervaert JW: Passive immunization with hypochlorite-oxLDL specific antibodies reduces plaque volume in LDL receptor-deficient mice. PLoS One. 2013 Jul;16;8(7):e68039.

Schreinemacher M, **Henatsch D**, van Barneveld K, Bouvy N: The need for standardised animal models and scoring systems in assessing mesh biocompatibility. Hernia. 2010 Jun;14(3):335-336.