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Cover photo by Jussi Sarin

I believe that professional wrestling is clean and everything else in the world is fixed.

Frank Deford

To my family

ABSTRACT

Jussi Sarin

Bioactive glass S53P4 and tissue adhesives in the surgical treatment of chronic middle ear and mastoidal infections

University of Turku, Faculty of Medicine, Department of Otorhinolaryngology – Head and Neck Surgery; Department of Biomaterials Science and Turku Clinical Biomaterials Centre; Doctoral Programme in Clinical Research

The treatment of chronic middle ear infection often includes surgery, which addresses areas of the middle ear and the mastoid cavity affected by the infection. Several factors favor the filling of the opened mastoid cavity after the so-called radical mastoidectomy. Many materials with different qualities have been used over the decades as mastoid obliteration materials, either by themselves or in a combined manner.

In the clinical part of this study, bioactive glass (BG) S53P4 particles were used to obliterate the mastoid cavity of 26 patients. The second and third part of this study examined the solubility and strength of fibrin glue-bioactive glass– and cyanoacrylate glue-bioactive glass –composites *in vitro*. Finally, a keratinocyte cell model was used to determine what kind of an effect bioactive glass S53P4 particles might have on the cells of ear cholesteatoma disease, or on the cells of normal skin of the surgical area.

Based on the results of this study, bioactive glass S53P4 as a mastoid cavity obliteration material produces a dry, safe ear, and may help to achieve a more normal appearance and function of the outer ear canal. Fibrin glue seems to be a suitable addition to BG granules, as it binds to BG granules making clinical use easier, but does not have a negative influence on the solubility process of BG. Cyanoacrylate glue, on the other hand, binds BG granules into a very solid composite structure that retains its strength in spite of a 30-day water exposure. It does not seem ideal to be used with BG granules in mastoid obliteration, but it might be a candidate for fixation of rigid BG composite implants. Based on the data of the *in vitro* cell model using immortalized HaCaT keratinocytes, BG S53P4 seems to inhibit the growth of keratinocyte cells and appears to trigger cell apoptosis with a direct cell-BG granule –contact. As these effects are constrained, BG S53P4 could have potential to reduce the likelihood of ear cholesteatoma recurrence, while not being unnecessarily harsh to normal skin.

Key words: Bioactive glass, S53P4, mastoid obliteration, fibrin glue, cyanoacrylate glue, cholesteatoma, HaCaT keratinocytes

TIIVISTELMÄ

Jussi Sarin

Biolasi S53P4:n ja kudosliimojen käyttö pitkäaikaisen välikorva- ja kartiolisäketulehduksen kirurgisessa hoidossa
Turun yliopisto, Lääketieteellinen tiedekunta, Korva-, nenä- ja kurkkutautioppi; Biomateriaalitieteen laitos ja Turun kliininen biomateriaalikeskus; Turun kliininen tohtoriohjelma

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Pitkäaikainen välikorvatulehdus edellyttää usein välikorvaan ja korvalokerostoon kohdistuvaa leikkaushoitoa. Useat seikat puoltavat leikkauksessa avatun korvalokeroston täyttämistä sopivalla materiaalilla, ja materiaaleja onkin vuosikymmenten mittaan ollut käytössä useita.

Tämän tutkimuksen kliinisessä osassa 26 potilaan korvalokerosto täytettiin S53P4 biolasirakeilla. Toisessa ja kolmannessa osatyössä selvitettiin fibriinikudosliiman ja syanoakrylaattiliiman liukenemista ja lujuutta biolasirakeiden kanssa yhdessä käytettynä. Viimeisessä osatyössä tutkittiin biolasirakeiden vaikutusta kokeellisessa keratinosyyttisolumallissa, jotta syntyisi käsitys biolasin mahdollisesta vaikutuksesta kolesteatoomatautiin ja ihon keratinosyyttisoluihin.

Tämän väitöskirjatutkimuksen perusteella bioaktiivinen lasi S53P4 sopii hyvin korvalokeroston täyttömateriaaliksi, ja sen avulla on mahdollista saavuttaa luotettavasti kuiva korva sekä mahdollisesti rakenteeltaan ja toiminnaltaan normaalia muistuttava korvakäytävä. Fibriiniliima soveltuu liukenemisominaisuuksiensa perusteella hyvin käytettäväksi biolasirakeiden kanssa. Syanoakrylaattiliiman huomattava lujuus ja liukenemattomuus eivät puolla kyseisen liiman yhteiskäyttöä biolasirakeiden kanssa korvalokerostossa, mutta kiinteiden biolasi-implanttien kiinnitysmenetelmänä syanoakrylaattiliima voisi tulla kyseeseen. Viimeisessä osatyössä käytetyn HaCaT keratinosyyttisolumallin perusteella biolasi S53P4 näyttää estävän keratinosyyttien kasvua ja altistavan välittömässä biolasikontaktissa olevat keratinosyyttisolut ohjelmoidulle solukuolemalle. Tämän havainnon perusteella korvalokeroston S53P4 biolasitäytöllä voisi olla rooli koleateatoomataudin uusiutumisen ehkäisemisessä, ilman merkittävää häiritsevää vaikutusta leikkausalueen ihon terveisiin keratinosyytteihin.

Avainsanat: Bioaktiivinen lasi, S53P4, korvalokeroston täyttö, fibriiniliima, syanoakrylaattiliima, helmiäiskasvain, HaCaT keratinosyytit

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ABBREVIATIONS

AOM = acute otitis media

BG = bioactive glass

CA-glue = cyanoacrylate glue

COM = chronic otitis media

CSF = cerebrospinal fluid

CSOM = chronic suppurative otitis media

CT = computed tomography

CWD = canal wall down

CWR = canal wall reconstruction

CWU = canal wall up

DMEM = Dulbecco's modified Eagle medium

DWI = diffusion weighed imaging

HA = hydroxyapatite

hBMSC = human bone marrow-derived mesenchymal stem cell

hMSC = human multipotent mesenchymal stromal cell

HRCT = high-resolution computed tomography

KGFR = keratinocyte growth factor receptor

KGM = keratinocyte growth medium

LCCA = long-chain cyanoacrylate

MEM-NEAA = minimum essential medium non-essential amino acids

MRI = magnetic resonance imaging

PEG = polyethylene glycol

pkg-1 = phosphoglycerate kinase-1

PTA = pure tone average

rhBMP = recombinant human bone morphogenetic protein

SBF = simulated body fluid

SCCA = short-chain cyanoacrylate

TCP = tricalcium phosphate

wt% = weight percent

β-TCP = beta-tricalcium phosphate

β-TPP = beta-tricalcium polyphosphate

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I   Sarin J, Grénman R, Aitasalo K, Pulkkinen J. Bioactive glass S53P4 in mastoid obliteration surgery for chronic otitis media and cerebrospinal fluid leakage. Ann Otol Rhinol Laryngol. 2012 Sep;121(9):563-9.

II   Sarin J, Björkvik L, Hiltunen M, Hupa L, Pulkkinen J, Vallittu PK. The effect of fibrin sealant on bioactive glass S53P4 particles – pH impact and dissolution characteristics in vitro. J Sci Adv Mat Dev. 2016 (1): 482-487.

III   Sarin J, Hiltunen M, Hupa L, Pulkkinen J, Vallittu PK. Compression properties and dissolution of bioactive glass S53P4 and n-butyl-2 cyanoacrylate tissue adhesive-composite. Biomed Mater Eng. 2016 Sep 28;27(4):425-436.

IV   Jussi Sarin, Minna Vuorenmaa, Pekka K. Vallittu, Reidar Grénman, Pia Boström, Jaakko Pulkkinen. The viability of HaCaT keratinocytes after exposure to bioactive glass S53P4 –containing cell culture media. Manuscript.

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1   INTRODUCTION

The purpose of ear surgery is to eradicate a specific disease from the affected ear, with a secondary aim to restore hearing or other loss of function, such as facial nerve paresis, caused by the disease. As the temporal bone is quite small with many important structures within, organ of hearing, balance end organ, facial nerve and internal carotid artery among them, special surgical equipment and training are needed. Among other necessary tools, an operating microscope can be considered as a prerequisite for modern ear surgery, as suggested by Simpson in 1947 at the proceedings of the Royal Society of Medicine (Simpson 1947). Today, it is difficult to imagine an otologist's practice without access to instrumentation offering adequate magnification. Since the early 1980's the microscope has been, indeed, a fundamental accessory not only in the operating room but also in the office setting (Saunders 1980).

In the treatment of chronic middle ear infection, a mastoidectomy operation is often indicated. In this procedure, air cells in the mastoidal cavity are drilled open thus enhancing aeration of the ear. Sometimes in the case of ear cholesteatoma, a radical mastoidectomy is performed, making eradication of the advanced disease possible via generous exposure and visibility. Many drawbacks with this type of surgery have been well recognized since the early 1900's, among them an additional hearing impairment and a need for frequent cleaning, and methods to avoid a large mastoid cavity have been under scrutiny since the 1920's (Peters 1924, Brown 1932).

As sometimes inadequate sufficiency and quality of patients' own tissues are taken into consideration, allogenous materials, either biological or synthetic in origin, have gained momentum in ear surgery. Although traditionally used as homogenous filler, a single mastoid cavity obliteration material typically cannot offer all the desired beneficial qualities, and compromises have to be made. From this perspective, the possibility to benefit from combined use of multiple biomaterials as composite structures, pursuing either more favorable tissue response or more fluent surgical procedure, has been the catalyst for this research.

2   REVIEW OF LITERATURE

2.1   Chronic middle ear infection

While acute middle ear infection, or acute otitis media (AOM), causes a significant number of health care visits with over 700 million cases globally each year and can also be described as a considerable financial burden, chronic otitis media (COM) has a lower incidence of 31 million cases each year (Monasta et al. 2012). The prevalence of COM is still, however, considerable: 65 to 330 million people are affected, with 60 % of the patients having a significant hearing impairment (Morris 2010). COM or chronic suppurative otitis media (CSOM) is characterized by a chronic inflammation of the middle ear and mastoid cavity. Clinically, this prolonged inflammation presents with recurrent or persistent ear discharge through an eardrum perforation or a tympanostomy tube. As there is no consensus regarding duration of symptoms, the diagnosis of CSOM is considered when ear discharge persists, in spite of treatment, for over 6 to 12 weeks. (Daniel 2012).

Etiological factors associated with CSOM include poor socioeconomic conditions and frequent upper respiratory tract infections (Morris 2010). The patients with CSOM usually have a mixed or polymicrobial infection with aerobic and anaerobic bacteria, with Pseudomonas aeruginosa being the most frequent pathogen. The microbial array differs considerably from AOM, where Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and several viruses are typically found (Tsilis et al. 2013). Usual aerobic bacteria in CSOM also include Staphylococcus aureus, while the most important anaerobic bacteria are Peptostrepto-coccus, Fusobacterium spp, Prevotella, and Porphyromonas spp (Daniel 2012). Fungal infection is also sometimes a causative factor in CSOM, especially in immuno-compro-mised or diabetic patients (Daniel 2012).

CSOM is not only a disease causing discomfort and hearing impairment with chronic eardrum perforation and ear drainage, but also can cause severe sequelae. Intratemporal complications include facial nerve palsy and inner ear infection, and among the most common intracranial complications in CSOM are brain abscess, meningitis, sinus thrombophlebitis, perisinus abscess, epidural abscess and intra-cranial hypertension (Tsilis et al. 2013, Sun and Sun 2014).

The diagnosis of CSOM is based on typical symptoms, careful otologic examination and computed tomography (CT) scan findings. The symptoms include otor-rhea (ear discharge), hearing loss in the affected ear, aural fullness and occasionally otalgia. More severe symptoms are present with complications, and these include facial nerve palsy, vertigo, nystagmus, headache and sensorineural hearing loss. In physical examination, otomicroscopy with careful suctioning of the ear

discharge is required, allowing bacterial cultures and view of the eardrum perforation, mucosal edema, possible ossicular lesions and possible cholesteatoma. Hearing evaluation with audiometric testing and with Rinne and Weber tests are also needed. In many instances, a high-resolution CT scan of the temporal bone provides invaluable additional information of the status of the middle ear and mastoid cavity. Furthermore, if there are signs of intracranial complications, magnetic resonance imaging (MRI) is able to increase the reliability of the diagnosis (Daniel 2012, Tsilis et al. 2013).

2.2 Cholesteatoma

Cholesteatoma is a cystic, noncancerous lesion in the temporal bone and it is derived from an abnormal growth of keratinizing squamous epithelium (Kuo et al. 2015[A]). There are two types of cholesteatoma. Congenital cholesteatomas are rare, forming 1 % of all cholesteatomas. Congenital cholesteatomas grow behind an intact tympanic membrane during early childhood. Acquired cholesteatomas form the bulk of all cholesteatomas, with an incidence of 9 to 12.6 cases per 100.000 adults and 3 to 15 cases per 100.000 children, originating from a defect or a perforation in the tympanic membrane (Kuo et al. 2015[A], Luers and Hüttenbrink 2016). Underdeveloped countries have a higher prevalence of cholesteatoma compared to developed countries, and prevalence among Caucasians is reported being the highest (Kuo et al. 2015[A]).

Despite being a known phenomenon for over three centuries, a thorough knowledge behind the pathogenesis of cholesteatoma is still lacking. In addition, no widely accepted or uniform clinical classification system has been adopted for different types of the disease. Four theories have been proposed for the pathogenesis of acquired cholesteatoma, and probably all these mechanisms have their place in the overall disease process. According to the retraction pocket theory, a poor Eustachian tube function leads to negative pressure in the middle ear, thus predisposing the pars flaccida of the tympanic membrane to retract. As the retraction pocket deepens, desquamating keratin accumulates in the pocket, leading to cholesteatoma (Kuo 2014). Migration theory implies that when there is a perforation of the tympanic membrane, the keratinizing squamous epithelium of the lateral layer of the tympanic membrane is able to migrate towards the middle ear via perforation, causing cholesteatoma. The theory of squamous metaplasia proposes that a metaplastic transformation of the middle ear mucosa into keratinizing epithelium takes place, without any tympanic membrane defect. Finally, basal cell hyperplasia theory sees keratin-filled microcysts, forming on the medial layer of the pars flaccida of the tympanic membrane, a starting point for cholesteatoma growth (Kuo 2014).
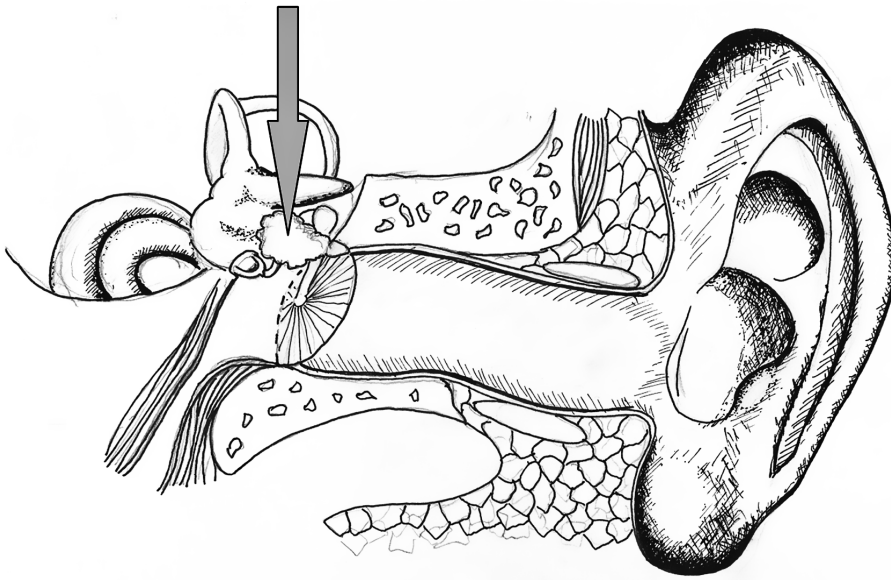
Figure 1. A cross-section image of human ear. An arrow points out an abnormal tissue growth (cholesteatoma) in the region of ossicles, medial to the tympanic membrane.

The diagnosis of cholesteatoma relies on patient history and clinical findings on otologic examination. Histopathologic examination, whether from the cholesteatoma samples obtained in an outpatient clinic prior to surgery or during ear surgery, are not routinely necessary, unless there is a concern for other pathology. In their study, Kircher et al. found a perfect agreement between the surgeons' findings during tympanomastoidectomy and pathologic diagnosis (Kircher et al. 2014). CT imaging and more specifically high-resolution computed tomography (HRCT) has been a useful addition demonstrating the presence of pathological soft tissue and possible bony erosions in middle ear and mastoid cavity (Corrales and Blevins 2013). While MRI has a poor resolution of bone anatomy, diffusion weighed imaging (DWI) sequences have demonstrated high sensitivity and specificity in identifying cholesteatoma (Corrales and Blevins 2013). Recently, Propeller (periodically rotated overlapping parallel lines with enhanced reconstruction) DWI sequences have appeared as especially useful tools in detecting cholesteatoma foci (Karandikar et al. 2015).

Untreated, cholesteatoma continues to grow over time destroying structures in contact with the cholesteatoma, and the disease tends to be especially aggressive in children (Visvanathan et al. 2012). Ossicular chain impairment leads to conductive hearing loss up to 60 dB. Ossicular chain involvement in cholesteatoma varies: erosion of the incus seems to be the most usual finding, whereas the destruction of the malleus and the stapes varies (Maresh et al. 2011, Franco-Vidal et al. 2014).

Advanced manifestations of cholesteatoma growth include progressive facial nerve palsy, a labyrinthine fistula presenting with vertigo and sensorineural hearing loss, even to the point of deafness. Intracranial complications, should the disease progress even further, include meningitis, encephalitis and brain abscess (Luers and Hüttenbrink 2016). Cholesteatoma can also grow laterally, extending to temporoparietal scalp (Zhu et al. 2014).

Infrequent cholesteatoma varieties include rare ear canal cholesteatoma, typically causing diagnostic challenge to physicians unfamiliar with the disease (Dubach et al. 2010). Unusual cholesteatoma occurrence has been reported after several months following a lightning strike blast injury, with a tympanic membrane rupture as an immediate finding (Scalzitti and Pfannenstil 2014). Misplaced squamous epithelium and cell debris can also situate unrelated to the ear, as in the case of intracranial epidermoid tumors (Sabin et al. 1987, Aboud et al. 2015).

The costs of cholesteatoma treatment are significant. Roche et al. reported the average hospital charge per patient per year being in the vicinity of 10 000 U.S. dollars (Roche et al. 2013). In spite of active research effort, there have been no conservative treatment options available, and only recently a promising treatment target via keratinocyte growth factor receptor (KGFR) inhibition has been published (Yamamoto-Fukuda et al. 2014). Surgery has remained the curative treatment of choice (Kuo 2014, Kuo et al. 2015). After surgical treatment, adequate follow-up of the patient is necessary owing to the recurrent nature of the disease. Recidivism rates are, to large extent, dependent on the surgical technique of choice and the experience of the surgeon, and varies between 9–25 % (Charachon et al. 1991, Visvanathan et al. 2012, Kuo et al. 2014, Neudert et al. 2014). Recidivism of cholesteatoma can originate either from the residual cholesteatoma cells, or re-occurrence of predisposing factors, such as the retraction of the tympanic membrane, can lead to disease recurrence. Recidivism is usually evident in a matter of a few years, but the disease might occur as late as 16 years after primary surgery, as reported by Kuo et al. with their series of 146 pediatric patients (Kuo et al. 2014). Fortunately, early recidivism of the disease has become easier to evaluate due to progress in MRI techniques, as discussed earlier, allowing detection of residual or recurrent cholesteatomas down to 3 mm in size. Hence, the routine need for the so-called second-look surgery is likely to decrease, unless a second operation addressing conductive hearing loss is needed in later phase.

2.3   Treatment of chronic middle ear infection

Different treatment options for CSOM lack, as a whole, strong quality of evidence, as there usually are few high quality studies comparing treatment modalities with placebo (Morris 2010). The goal is to eradicate the infection, stop the otorrhea and

eventually heal the tympanic membrane while avoiding recurrence (Daniel 2012). Ear cleansing, preferably under the microscope, is considered an integral part of any treatment protocol for chronically running ear. Thereafter, local medication is able reach the middle ear space proficiently via tympanic membrane perforation. Topical antibiotic-corticosteroid -combination may improve symptoms and reduce otorrhea, especially when combined with removal of ear canal debris, while systemic antibiotic therapy seems less effective (Morris 2010). As topical antibiotics reach very high tissue concentration at the site of infection compared to those of systemic antibiotics, they should obviously be preferred as the first-line treatment (Daniel 2012). Due to their effectiveness and safety profile, fluoroquinolones are preferred (Daniel 2012). With the use of topical antibiotic therapy, the emergence of bacterial antibiotic resistance is very unlikely, as the concentration in the ear canal is well above the mean inhibitory concentration for different bacteria. There is a low quality evidence to support the use of systemic antibiotic therapy at the time of mastoidectomy, while refraining antibiotic therapy with surgery seems a less effective treatment decision (Morris 2010).

A common situation with surgical treatment of various diseases is the low quality of evidence, as far as selected operative methods or operative treatment *per se* are concerned. For surgery of CSOM with or without cholesteatoma, the level of quality of evidence is low (Morris 2010). Adding to the challenge of reliably comparing different surgical methods, most articles fail to formally stage the disease or report recurrence rates as Kaplan-Meier disease-free curves (Mor et al. 2014). The range of surgical treatment of CSOM varies: simple closure of the tympanic membrane defect might suffice after an adequately long dry period of the affected ear, whereas sometimes extensive surgery of the temporal bone and beyond might be required, including mastoidectomy and ossiculoplasty. Although some cholesteatoma patients, unfit for surgery, might be treated with regular debridements on outpatient visits, no definite conservative treatment options for cholesteatoma exist (Luers and Hüttenbrink 2016). During surgical treatment, the diseased region in the ear must be adequately exposed by drilling away the surrounding bone, followed by removal of the cholesteatoma completely. Cholesteatoma surgery might be considered somewhat analogical to cancer surgery: only one missed cholesteatoma cell could lead to re-occurrence of the disease (Luers and Hüttenbrink 2016). Eradication of disease and prevention of recurrence are among primary goals, while preservation or improvement in hearing is, although important, a secondary objective. With increasing surgical experience, recurrence rates for cholesteatoma are lower (Stankovic 2013).

In mastoidectomy, a retroauricular skin incision is made and air cells in the mastoidal cavity are drilled open in an attempt to create a smooth, self-cleaning cavity. With cholesteatoma, the disease is removed after gaining sufficient exposure

in the affected region. If possible, posterior ear canal wall is preserved as a natural barrier between the mastoid cavity and ear canal, making this approach the canal wall up (CWU) mastoidectomy (Bennett et al. 2006). However, the nature of the disease sometimes requires greater visibility compared to the CWU method, necessitating an open cavity tympanomastoidectomy or the canal wall down (CWD) mastoidectomy approach (Bennett et al. 2006). In such a case, an adequately large ear canal must be also formed by means of meatoplasty, in order to allow for sufficient aeration of the ear, epithelization of the cavity and easier postoperative care (Bennett et al. 2006, Harun et al. 2015). The sinodural angle seems to be an especially important site for meticulous surgical attention, as this region often contains persistent air cells leading to additional surgery (Harun et al. 2015).

Intratemporal complications secondary to cholesteatoma include facial nerve paralysis and labyrinthine fistula. In the case of facial nerve paralysis, decompression of the facial nerve with cholesteatoma eradication is preferred, whereas labyrinthine fistula requires cholesteatoma matrix removal, followed by closure of the labyrinthine defect (Prasad et al. 2013). When CSOM is associated with serious intracranial complications, such as a brain abscess, rapid mastoidectomy in addition to abscess drainage seems to result in a mortality rate as low as zero percent (Sun and Sun 2014). In serious intracranial infections, antibiotic and corticosteroid therapy is obviously included (Prasad et al. 2013). Skull base osteomyelitis is a possibility with cholesteatoma, and preoperative HRCT and MR images should be carefully reviewed in order to avoid major postoperative cranial nerve complications (Lee at al. 2016).

As of late, there has been an effort towards minimally invasive surgery with the help of transcanal ear endoscopy. Hanna et al. reported success in acquiring adequate exposure during cholesteatoma surgery, without the need for CWD mastoidectomy, using endoscopic techniques combined with CWU mastoidectomy. The recurrence rate of cholesteatoma with an endoscopic technique was, however, higher compared to that with an open technique: 4% and 2 %, respectively (Hanna et al. 2014). Kuo et al. address the increased risk of residual cholesteatoma in their review, while also discussing the technical challenges associated with this form of ear surgery, among them the need for one-handed surgery and limitations in the endoscopic accessibility of the mastoid cavity (Kuo et al. 2015[B]). Use of ear endoscopes also puts additional requirements on other instrumentation. For example, Yau et al. found the use of curved fiberoptic laser probes useful for precise removal of cholesteatoma with an endoscopic technique (Yau et al. 2015).
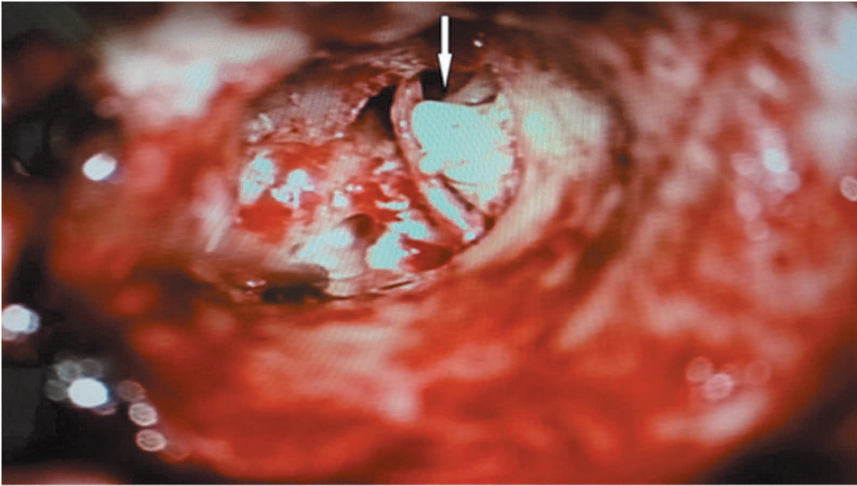
Figure 2. Middle ear cholesteatoma of a child. Tympanic membrane has been lifted anteriorly and chorda tympani nerve has been dislocated in a caudal direction preceding cholesteatoma (pointed by arrow) removal. Photo: Jussi Sarin.

2.4   Mastoid cavity obliteration surgery

There are several reasons to avoid CWD mastoidectomy. An open mastoid bowl requires perpetual cleaning (Bennett et al. 2006, Harun 2015) and is usually intolerant to water (Alves et al. 2016). The postoperative hearing level is worse after the CWD technique due to greater conductive hearing impairment, compared to preserving or reconstructing a more anatomical structure of the ear canal wall (Lee et al. 2009, Osborn et al. 2012). In CWD mastoidectomy, when an open cavity is created, the acoustic resonance of outer ear is severely altered. In fact, the average resonance frequency was reduced by more than a 1000 Hz in a series of 20 patients published by Hartwein, an important consideration for maintaining speech perception as good as possible (Hartwein 1992). Compared to an open mastoid cavity with resulting large ear canal volume, an obliterated mastoid cavity produces higher resonance frequencies, leading to better function in regards to hearing (Jang 2002). Overall, mastoid obliteration seems to not only improve hearing and reduce ear drainage (Leatherman and Dornhoffer 2004), but also improve the quality of life (Dornhoffer et al. 2008). Preferable qualities in a good mastoid cavity filler material include biocompatibility, resistance to infections, the ability to maintain sufficient material volume over time and easy removal, should there be a need for revision surgery (Yung and Bennett 2013).

In 1911, Harris Mosher described a method of using a local soft tissue flap to cover a bony defect in mastoid cavity, after performing mastoidectomy for acute mastoiditis (Mosher 1911). Popper included temporalis muscle and periosteum for his flap choice (Popper 1935). Palva used additional bone paté with a soft tissue flap

in order to obliterate the mastoid (Palva 1973), and later recommended mastoid obliteration with bone chips and bone paté, with a meatally based musculoperiosteal flap in all mastoid surgery (Palva 1979). With time, several new techniques and obliteration materials have emerged, with the aim to combine the advantage of supreme exposure of CWD technique with the minor functional handicap of the CWU technique (Vercruysse et al. 2008, Yung and Bennett 2013).

Recently, methods which avoid the need for mastoid obliteration, while still utilizing the CWD technique, have been published. Van der Gucht and colleagues removed the posterior ear canal wall temporarily during cholesteatoma surgery using an oscillating saw. Later, the posterior wall was fixed in an anatomically correct position with titanium microplates and screws, when possible (Van der Gucht et al. 2014). Walker et al. were very pleased with the excellent intraoperative visibility and maintenance of near normal anatomy postoperatively offered by the canal wall reconstruction (CWR) technique, but it also contained risks: 14 (4.9 %) of their total of 285 ears had an intraoperative cerebrospinal fluid (CSF) leak owing to the use of an oscillating saw or the standard rotating burr (Walker et al. 2014).

2.4.1 Autologous mastoid cavity obliteration materials

In mastoid cavity obliteration surgery, Yung and Bennett have emphasized the importance of adequate soft tissue flap coverage of the filler materials, in order to cover the whole reconstructed ear canal area (Yung and Bennett 2013). Random regional soft tissue flaps, comprising of periosteum and muscle tissue, form the basis of autologous mastoid cavity obliteration materials. Autologous bone, either as bone chips or bone paté, has been a popular filler choice (Yung and Bennett 2013). Although naturally biocompatible, volume loss has been a hindrance with bone, while cartilage with its low metabolism maintains its bulk better over time and has also served as a filler material with pediatric patients (Kuo et al. 2014). As with many patient-derived biomaterials, an adequate amount of filler tissue is often difficult to harvest, and harvesting typically causes additional morbidity at the harvest site. Postauricular flaps remain, in conjunction with autologous bone, an effective technique as published by Ramsey et al. with their success rate of 82 % in achieving a small, dry mastoid cavity (Ramsey et al. 2004). A systematic review by Alves et al. compared results from CWD mastoidectomy and following mastoid obliteration with autologous bone. They included a total of 1017 cases with a minimum follow-up of 12 months: 78-100 % of patients achieved a dry ear, with cholesteatoma recurrence rates between 0 % and 17 % (Alves et al. 2016).

Fat tissue, as an autologous obliteration material, is usually found in generous amounts. Kos and colleagues reported a series of 46 patients, for whom mastoid obliteration with fat tissue, i.e. Rambo operation, resulted in a dry ear ending the need for frequent debridement for most of the patients. However, 7 patients still

had recurrent infections and three patients developed residual cholesteatoma (Kos et al. 2006).

2.4.2   *Allogenous mastoid cavity obliteration materials*

Allogenous mastoid cavity obliteration materials can be divided into two groups: biological and synthetic. Demineralized bone matrix represents a non-synthetic, allogenous obliteration material, as used by Leatherman and Dornhoffer (Leatherman and Dornhoffer 2004). Lee et al. utilized allogenous cancellous bone chips as a cavity filler, acquiring good results in reconstructing an ear canal wall after CWD mastoidectomy (Lee et al. 2009).

Synthetic mastoid cavity obliteration materials seem to offer several advantages over both autologous and allogenous biological obliteration materials. The availability of a synthetic filler is, at least in theory, unlimited and there is no need to harvest the material either locally from the surgical site, or elsewhere causing additional morbidity to the patient while prolonging surgery. Allogenous biological obliteration materials, unlike synthetic materials, carry the inherent risk of microbial transmission to the patient. Synthetic obliteration materials, however, can often be expensive.

In 1991, Charachon and colleagues reported the possibility to use either bone paté or synthetic ceramic granules with Palva flap in reconstruction after radical mastoidectomy (Charachon 1991). In the synthetic calcium phosphate bone graft group, hydroxyapatite (HA) has served, in granule form, as a suitable cavity filler material (Yung 1996, Mahendran and Yung 2004). However, experiences with HA bone cement are poor, often necessitating revision surgery due to infectious (Mahendran and Yung 2004) and severe osteitis complications (Ridenour et al. 2008). Granular beta-tricalcium phosphate (β-TCP) has been used in mastoid cavity obliteration in a series of 13 patients. On follow-up, CT examination revealed that the obliteration material granules dissipate slowly over the years and no complications were observed (Minoda et al. 2007). In their combined human and animal study, Lee at al. used β-TCP as an obliteration material in 20 CWU mastoidectomy patients, and found one case of postoperative infection necessitating a second operation (Lee et al. 2013). In the animal study part, on the other hand, they used beta-tricalcium polyphosphate (β-TPP) as cavity filler in otic bullae and skull bone defect in rats, and reported new bone formation in histological and radiological studies, suggesting β-TPP as a treatment option in mastoid cavity obliteration. Kakigi et al. used, after CWD mastoidectomy, calcium phosphate paste as an obliteration material, and proceeded to cover the paste with an artificial dermis soaked with basic fibroblast growth factor. All cavities were decreased in volume, leading the authors to state their method effective in mastoid cavity obliteration surgery (Ka-

kigi et al. 2009). Finally, calcium phosphate –based materials have also been combined: Franco-Vidal and colleagues used a ceramic biomaterial consisting of 60% HA and 40% β-TCP, and found the material effective and well-tolerated (Franco-Vidal et al. 2014).

Contradictory results have been published with glass ionomeric cavity fillers. Ionocem (IONOS, Germany) -glass ionomeric cement seems inappropriate as a mastoid cavity filler material, as adverse tissue reactions with severe infection and extrusion of the material from the cavity are very common (Kupperman and Tange 2001). On the other hand, SerenoCem™ -glass ionomeric cement granules, mixed with cefuroxime and blood with Tisseel fibrin glue fixation, produced clearly better long term results with fewer complications (Clark and Bottrill 2007).

An argument can be made that there is no need for a synthetic mastoid cavity filler to be bioactive. The inertness of the material, i.e. tissue compatibility, will suffice. Such is the case with an inexpensive mastoid cavity silicone block obliteration material, as published by Cho et al. in their series of 20 patients (Cho et al. 2012). However, few allogenic materials are devoid of the capability to produce a foreign body reaction *in vivo* (Al-Maawi et al. 2017). As Cho et al. had to re-operate one of their patients due to a recurrent cholesteatoma 37 months after the first surgery, granulation tissue was observed and removed from the silicone block -obliterated cavity. The modern research of medical biomaterials has pushed material development towards bioactive products, where materials would stimulate new bone formation while slowly reabsorbing at the surgical site, preferably offering other beneficial qualities as well.

Recently, Skoloudik and colleagues published their results with a composite biomaterial consisting of human multipotent mesenchymal stromal cells (hMSCs), hydroxyapatite and tissue glue and found hMSCs to have a beneficial effect on new temporal bone formation in a guinea pig model (Skoloudik et al. 2015). The idea of combining several biomaterials as cavity filler is not new: Nishizaki et al. used bone morphogenic protein-2/collagen composites in rat mastoids, and saw newly formed bone with near-normal bone structure histologically, with no adverse reactions (Nishizaki et al. 2003). Indeed, the future of mastoid cavity obliteration materials might well lie in composite structures and tissue engineering, as typically no single biomaterial can supply all the desirable qualities (Table 1).

Table 1. Different types of mastoid cavity obliteration materials that have been in clinical use.

	Mastoid cavity obliteration materials	safety	availability	resorption	special properties	outcome in published studies	mean follow-up and range
autologous	musculocutaneus / pericranial flap	safe	compromised with re-operations	muscle loses volume	perfect biocombatibility as autologous grafts, without any foreign body reactions	dry, small cavity in 82–90 % of patients	2.5 years (1—7)
	fat	safe	good	intermediate		dry ear in 85 % of patients, good potential to reduce cavity size after CWD	8 years (1—23)
	cartilage	safe	limited	low		79 % of paediatric patients without cholesteatoma recidivism	12 years
	bone	safe*	limited	bone paste loses volume		dry ear in 78—100 % of patients, cholesteatoma recidivism in 0—12 %	3—6 years
allogenous	demineralized bone matrix	microbiological contamination possible	limited	volume loss possible		dry ear in 73 % of patients at 9 weeks, 100 % dry at 28 weeks	14.5 months (6—20)
	allogenous bone chips	microbiological contamination possible	limited	low		reconstructed canal wall maintained its cylindrical shape in 91 % of patients	33 months (12—54)
	hydroxyapatite granules	safe	unlimited	low		small, dry cavities in 97 % of patients	1—5 years
	hydroxyapatite cement	unsafe	unlimited			causes infection including osteitis; 50—100 % of patients require revision and cement removal	
	glass ionomer cement	unsafe	unlimited			severe infections or material extrusion in 35 % of patients requiring revision surgery	1—5 years
	glass ionomeric granules	safe	unlimited	low	osteoinductive	a need for re-operation in 19 % of patients	2 years
	silicone blocks	safe	unlimited	no resorbtion	inert, cheap material	reconstructed canal wall maintained its cylindrical shape in 91 % of patients	49 months (6—90)
	β-tricalcium phosphate	safe	unlimited	intermediate		dry ear in 95—100% of patients	1 year
	bioactive glass granules	safe	unlimited	low	antibacterial, osteoinductive	dry ear in 97—100% of patients	2.2—4.8 years

* risk of introducing an infection or cholesteatoma into the obliterated cavity when patients' own bone paté is used, harvested from the primary surgical site

2.4.3 *Bioactive glass as a bone substitute and a mastoid cavity obliteration material*

Bioactive glass (BG), discovered almost 50 years ago, serves currently as an invaluable tool as a bone substitute. Since 1971, when Hench and colleagues found a particular glass composition to form a bond with bone (Hench et al. 1971) and not the usual surrounding fibrous capsule as a typical foreign body reaction, the overall understanding of BGs and BG-containing composite materials has vastly increased. Through rapid surface reactions, BGs consisting in varying percentages of Na_2O, CaO, P_2O_5 and SiO_2, form a biologically active hydroxyapatite surface layer, masking the material from immune cells *in vivo* (Figure 2). From there, the mineralization process continues through cell attachment and extracellular matrix production and new bone is formed (Hench and Jones 2015). Although hydroxyapatite and other types of calcium phosphate have often been a clinically preferred biomaterial owing to the challenges in BGs sintering processes, glass composition and –structure can be tailored currently to meet the clinical demands (Jones 2013).

Currently, there are two pure commercial formulations of BG available approved for clinical use: original discovery of Hench, 45S5 Bioglass (NovaBone, NovaBone Products, FL, USA) and S53P4 (BonAlive, BonAlive Biomaterials, Turku, Finland). The composition of 45S5 Bioglass is 45 weight percent (wt%) SiO_2, 6 wt% P_2O_5, 24.5 wt% CaO and 24.5 wt% Na_2O, whereas the percentages of S53P4 are 53 wt%, 4 wt%, 20 wt% and 23 wt%, respectively. As the silica content of these two products differs, so does the bioactivity: S53P4 is expected to have a slower bone-forming ability owing to the higher silica percentage compared to 45S5 (Hench and Jones 2015). As BGs dissolve, Ca-ions are released into the solution from the glass surface, while H^+ ions are exchanged from the fluid (Vallittu et al. 2015). This leads to a rapid increase in the pH of the solution, which in turn inhibits the growth of bacteria (Zhang et al. 2009). Indeed, BG S53P4 has antimicrobial properties towards a vast group of both aerobic (Munukka et al. 2008) and anaerobic (Leppäranta et al. 2008) bacteria. A particularly important observation is the ability of S53P4 to suppress the growth of methicillin-resistant Staphylococcus aureus (Munukka et al. 2008). Not only multiresistant bacterial strains, but also biofilm-producing bacteria are especially difficult to eradicate through conventional means. Therefore, the recent work of Bortolin et al. is especially important: BG S53P4 is able to reduce the growth of multiresistant Staphylococcus epidermidis, Acinetobacter baumannii and Klebsiella pneumoniae on titanium discs *in vitro* (Bortolin et al. 2016). Thus, the earlier study by Coraça-Huber et al. gained support, where Staphylococcus aureus biofilms grown on titanium discs were suppressed by BG S53P4 (Coraça-Huber et al. 2014). Drago et al., after showing the antibiofilm activity of S53P4 towards methicillin-resistant Staphylococcus aureus and multi-drug-resistant Pseudomonas aeruginosa, state that BG S53P4 has potential in treating prosthetic infections related to biofilms (Drago et al. 2014).
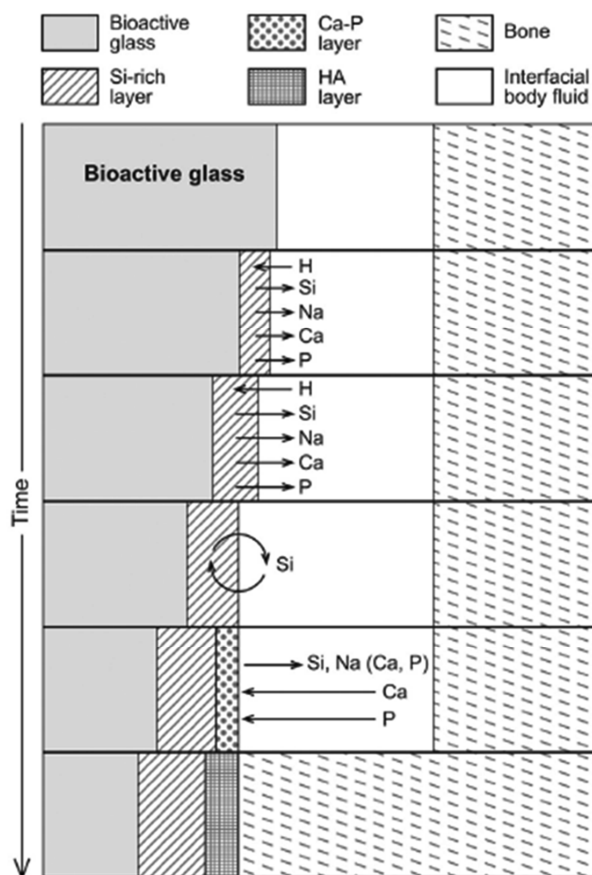
Fig. 3 – Schematic drawing of the dissolution of bioactive glass in bone.

Figure 3. Republished with permission from Vallittu PK, Närhi TO, Hupa L. Fiber glass-bioactive glass composite for bone replacing and bone anchoring implants. Dent Mater. 2015 Apr;31(4):371-81.

In addition to studies performed *in vitro*, there is strong clinical evidence to support the antibacterial properties of BG S53P4 (Aurégan and Bégué 2015). This feature, combined with the ability of new bone formation (Jang et al. 2007) and overall safety and biocompatibility of BGs (Wilson et al. 1981), has paved the way for BGs to be used as a bone cavity filling material in various clinical situations. In orthopedic surgery with benign bone tumors, BG S53P4 has been used as bone graft substitute (Lindfors et al. 2010). Chronic osteomyelitis, a particularly challenging-to-treat chronic infection of bone, has been successfully treated combining debridement surgery with BG S53P4 cavity filling in the limb and the spine area (Lindfors et al. 2010, McAndrew et al. 2013, Kankare and Lindfors 2016). In maxillofacial surgery, BG S53P4 has served as a cavity filler material in frontal sinus obliteration (Peltola et al. 2006), facial bone defect and orbital wall reconstruction

(Suominen and Kinnunen 1996, Peltola et al. 2008) and as a graft material in septal perforation repair (Stoor et al. 2001, Stoor and Grénman 2004). Lately, as a part of individualized fiber-reinforced composite implants, BG S53P4 has been used in both adult and pediatric populations suffering from cranial bone defects (Aitasalo et al. 2014, Piitulainen et al. 2015).

The idea to use bioactive materials in ear surgery gained momentum after Reck's work, in which favorable results of posterior canal wall reconstruction with bioactive glass-ceramic Ceravital were published (Reck 1984). In addition, Reck found this glass-ceramic material to be well tolerated in direct contact with tympanic membrane, as used for reconstruction of ossicular chain, and producing better postoperative audiological results compared to those of allogenous ossicular grafts. Jang et al. used 45S5 NovaBone particles in guinea pig otic bullas as a cavity filler material, and found no signs of infection or implant exposure. New bone formation, with both CT and histological confirmation, was observed at the end of the study after five months, as well as a definite bond between the implant and bone interface (Jang et al. 2007). In 2010, Stoor et al. were the first to publish clinical results of mastoid cavity obliteration with BG S53P4 granules after CWD surgery, with seven patients (Stoor et al. 2010). After 16 operations, Silvola reported his favorable experiences with mastoid obliteration using also BG S53P4 granules. Fourteen patients went through CWD mastoidectomy and two CWU mastoidectomy, suffering from continuous ear discharge after simple mastoidectomy (Silvola 2012). So far, the most comprehensive clinical experience has been published by Schimanski and Schimanski: their results of 843 mastoid obliterations include 133 patients with mastoid obliteration using BG S53P4 granules. BG obliteration produced very favorable results, as only 3 % of the patients showed abnormal postoperative findings, for example retraction of the posterior canal wall (Schimanski and Schimanski 2015).

When the surface reactions of BG in the surrounding liquid environment are considered, the question of inner ear safety arises. Bernardeschi and colleagues published their results with 41 cases, where BG S53P4 was used as a mastoid and epitympanic obliteration material. They found no signs of labyrinthine complications, in the form of either complaints of vertigo / dizziness or postoperative sensorineural hearing loss among the patients. Furthermore, they noticed no adverse skin reactions from the obliteration material in the follow-up period after three months; up until that time, the skin of the posterior ear canal wall showed signs of inflammation with swelling, warranting the use of ear wick and antibiotic-corticosteroid ear drops (Bernardeschi et al. 2015).

2.5   **Animal models of cholesteatoma**

Since the 1980's, when Chole et al. reported an incidence of nearly 50 percent of spontaneous aural cholesteatomas in adult Mongolian gerbils, and Feinmesser and colleagues reported a propensity of sand rat to develop spontaneous cholesteatomas, animal cholesteatoma models have been an invaluable research tool (Chole et al. 1981, Feinmesser et al. 1988). The Gerbilline cholesteatoma model has a resemblance to human cholesteatoma in several aspects, as it is histologically similar to human cholesteatoma and erodes bone with a potential to invade into the labyrinth and intracranial space (Chole et al. 1981). However, as multiple biological markers of cell differentiation, cell adhesion and inflammation differ between gerbilline and human cholesteatoma disease, this popular model is not 100 % valid (Ghoufani et al. 2007).

As reviewed by Yamamoto-Fukuda and colleagues, a large variety of different animal models exists: in addition to Mongolian gerbil, also mouse, Guinea pig, chinchilla and rat have all been used (Yamamoto-Fukuda et al. 2011). Different cholesteatoma induction methods, on the other hand, offer a possibility to scrutinize different theories of cholesteatoma pathogenesis, as discussed in chapter 2.2. In Mongolian gerbil, surgical ligation of the external auditory canal produces, with a high success rate, a cholesteatoma capable of bone erosion (McGinn et al. 1982). This model is based on the accumulation of keratin debris from the ear canal skin, as debris is incapable of exfoliating outside the ear due to canal ligation. Furthermore in the same species, Eustachian tube obstruction by means of electrocautery is another option of cholesteatoma induction, although with a lower success rate (Kim and Chole 1998), as summarized in Table 2. According to the retraction pocket theory, Eustachian tube dysfunction leads to a retraction of the Shrapnell's membrane, thus provoking acquired cholesteatoma development (Yamamoto-Fukuda et al. 2011). Finally, a third method of cholesteatoma induction in Mongolian gerbil is the application of an irritating chemical substance, such as propylene glycol, in the middle ear. In comparison, this method turned out to be the least reliable in Kim's and Chole's work (Kim and Chole 1998). An old analogy of cholesteatoma, "skin in the wrong place", was the basis of Si et al's work: through tympanic membrane, an autologous skin graft was placed into the middle ear in mice, while also injecting Pseudomonas aeruginosa into the tympanum (Si et al. 2013). After six weeks, the cholesteatoma rate was 92 % in the operated ear.

A complex cholesteatoma model, published by Yamamoto-Fukuda and colleagues, offers a possibility to understand the pathogenesis of the disease more comprehensively (Yamamoto-Fukuda et al. 2010). The pars flaccida of the tym-

panic membrane was removed from male Mongolian gerbils. The defect was repaired with corresponding portion of the tympanic membrane of female gerbil, followed by ear canal ligation to induce cholesteatoma. In the control group, only ear canal ligation was performed, without a myringoplasty procedure. As a result, authors reported cholesteatomas in all ears in each group. With in situ-PCR technique, X chromosome-linked phosphoglycerate kinase-1 (pkg-1) gene was searched in the cholesteatoma paraffin sections. In the hybrid model group with implanted allogenous tympanic membrane portions, two pkg-1 spots were observed in the epithelial nuclei, leading the authors to conclude that the tympanic membrane itself was the origin of cholesteatoma epithelial cells, not ear canal skin or cells in the tympanic cavity.

Not only does the use of an animal model of cholesteatoma permit a pathogenesis research of the disease, but it also allows intervention study protocols to take place. Unfortunately, several different methods of controlling or curing the disease have failed in animal models: intratympanic administration of mitomycin C (Melo et al. 2013), topical hyaluronic acid on top of the tympanic membrane (White et al. 1995), systemic cyclophosphamide (Pownell et al. 1994) and isotretinoin (Jove et al. 1990) were all ineffective in cholesteatoma prevention. The drug 5-fluorouracil, widely used in cancer chemotherapy, has been shown to have some potential in reducing the proliferation of tympanic membrane epidermis and hyperplasia of the connective tissue layer, followed by a reduced likelihood of cholesteatoma development (Wrigth et al. 1991). In addition, topical use of trans-retinoic acid in a guinea pig model shows some promise as it decreased cholesteatoma rates from 75 % in the control group to 30 % in the treatment group, respectively (Antunes et al. 2008). While mastoid obliteration with plaster of Paris prevented experimental cholesteatoma formation in guinea pigs (Hinohira et al. 1998), obliteration-induced severe inflammation confines this method as clinically unthinkable. However, as the definite clinically relevant conservative treatment method of cholesteatoma is so far only a wish, the strongest published potential seems to exist in selective keratinocyte growth factor receptor inhibitor SU5402, which completely prevented cholesteatoma formation in a rat model (Yamamoto-Fukuda et al. 2014).

Table 3. *In vitro* - cholesteatoma models

In vitro - model	Origin of keratinocytes	Culture medium	Antibiotics	Other supplements	Advantages	Disadvantages
Monolayer cholesteatoma models	surgical specimens during ear surgery	Dulbecco's modification of Eagle's medium (DMEM)	penicillin 100 IU/ml, streptomycin 100 µg/ml	10% fetal bovine serum, glutamine 0.4 mg/ml	2 week-culture period	large cell growth variation
	surgical specimens during ear surgery	keratinocyte serum-free medium (SFM)	penicillin 500 IU/ml, streptomycin 500 µg/ml, 5 µg/ml amphotericin B	2.4 U/ml Dispase II and 0.2% trypsin digestion of the specimens before culture	not specifically discussed	not specifically discussed
	surgical specimens during ear surgery	keratinocyte-SFM	penicillin, streptomycin, amphotericin B ad 1%	0.25% trypsin digestion of the specimens before culture		
	surgical specimens during ear surgery	alpha-modification of minimum essential medium	penicillin 50 IU/ml, 50 µg/ml streptomycin	5% fetal bovine serum	80% of specimens succesfully cultured	large cell migration variation
3-D cell models	commercial human dermal keratinocytes	keratinocyte-SFM	not specified	fibroblast layer grown separately	benefits of a three-dimensional tissue structure	complex model with air-liquid interface
	HaCaT immortalized keratinocytes	DMEM, 10% fetal bovine serum, glutamine				complex extracellular matrix model
	surgical specimens during ear surgery	keratinocyte-SFM	gentamicin 5 mg/ml	in DMEM		complex model with air-liquid interface

Table 2. Animal models of cholesteatoma .

Animal model	Method of cholesteatoma induction	Timespan	Success rate	Advantages	Disadvantages
Mongolian gerbil	surgical ligature of external ear canal	6—9 months	up to 100%	good experimental model for the study of bone erosion, easy	
	eustachian tube blocking with electrocautery	4 months	ca. 75%		technically demanding
	transplantation of allogenous piece of tympanic membrane	2 years	up to 100%	model for the study of the epithelial cell origin	technically demanding, time consuming
guinea pig	skin graft placement into middle ear	2 months	ca. 90%	rapid, strong inflammatory reaction	no bone erosion
mouse	skin graft placement into middle ear with Pseudomonas injection	6 weeks	92%	technically easy	
	calvarial placement of skin and fur keratin particles	1—4 weeks	not available	good model for inflammatory osteolysis	not a direct ear model
chinchilla	injections of propylene glycol into middle ear	4 weeks	60—70%	good model for drug administration studies	

2.6   In vitro cholesteatoma models

Since the 1980's, the possibility to utilize epithelial cell cultures in cholesteatoma research has been acknowledged (Proops and Parkinson 1983, Proops et al. 1984). Minotti and colleagues used cholesteatoma matrix from patients, cut into small fragments and grown in alpha modification of minimum essential medium, and studied the inhibitory effect of all-trans retinoic acid (Minotti et al. 1996(A)) and low calcium levels (Minotti et al. 1996(B)) on the rate of cell migration *in vitro*. Several other authors have also published their *in vitro* cholesteatoma models, as summarized in Table 3. Commonly, these models use patient cholesteatoma samples as a starting point, and the usual goal is to produce a monolayer of keratinocyte cells for either studies on the disease pathology or in order to test a specific intervention theory (Kobayashi et al. 2005, Sedlmaier et al. 2005, Helgaland et al. 2010).

Helgaland and colleagues produced their monolayer cholesteatoma model using cholesteatoma matrix from 10 patients undergoing ear surgery, and their model represents a successful experiment using primary cholesteatoma samples (Helgaland et al. 2010). Surgical specimens were first rinsed with saline solution, and 1 mm circular samples, cut using a needle, were placed directly in 24-well culture plates. Dulbecco's modified Eagle medium (DMEM) with 10 % fetal bovine serum supplementation was used as a culture medium. Glutamine, penicillin and streptomycin were added in concentrations of 0.4 mg/ml, 100 IU/ml and 100 μg/ml, respectively. A 5 % CO_2 atmosphere was used at 37 °C during incubation. Keratinocyte culture growth was observed and cell area was calculated based on light microscopy images. Subsequently, 12 to 14 days after initial culture, analysis of cytokine secretion in conditioned medium samples was undertaken for interleukin 6, tumor necrosis factor and monocyte chemoattractant protein-1.

In an effort to create an *in vitro* cholesteatoma model, preferably capable of mimicking *in vivo* conditions more accurately compared to monolayer cell models, Tanaka and colleagues published their results with a three-dimensional cholesteatoma model in 2005 (Tanaka et al. 2005). Cultured skin equivalents were used for this purpose: commercial human epidermal keratinocytes, grown initially in a serum-free keratinocyte growth medium (KGM), were placed on top of a layer of human dermal fibroblasts, grown initially in DMEM, respectively. In addition to fibroblasts, the basal portion of this layered structure consisted of a gel, manufactured to contain type I collagen and DMEM, supplemented with 10 % fetal bovine serum. The culture was maintained in an air-liquid interface, so that only the keratinocyte cell layer was exposed to air. This enabled a cornified epithelial layer to form, while the basal portion of the structure was submerged into a mixture of KGM and DMEM, supplemented with 5 % fetal bovine serum and additional Ca_{2+}

and 2-O-a-D-glucopyrasonyl-L-ascorbic acid. Upon completing the model, the effects of experimentally created epidermal and dermal defects were studied. While the authors stated this model to resemble natural skin in many aspects and being beneficial as far as studying migration patterns of epidermis are concerned, they emphasized the need for further model development in order to better mimic the proliferation of stratified squamous epithelium and accumulation of keratin debris, similar to actual cholesteatoma (Tanaka et al. 2005).

Later studies in the area of 3-D cholesteatoma models include a co-culture of nontumorigenic immortalized HaCaT keratinocytes with normal human dermal fibroblasts, as published by Laeeq and Faust (Laeeq and Faust 2007). In this model, both cell types were cultured on an artificial extracellular matrix, consisting of gelatinous protein mixture Matrigel and DQ-collagen IV. As a result, the potential of fibroblasts to stimulate keratinocytes towards a more aggressive nature with proteolytic / invasive phenotype, were observed. So far, the only three-dimensional cholesteatoma model, where cholesteatoma keratinocytes from surgical samples were incorporated with a feeder layer of cholesteatoma fibroblasts, was described by Raynov et al. (Raynov et al. 2008). Fibroblasts were grown on a polyester membrane, and keratinocytes were added on top of this basal cell layer, utilizing an air-liquid interface system in the style described earlier by Tanaka and colleagues (Tanaka et al. 2005). The characteristic flaw of this model is understandably the inherent variation between different patient samples, and thus variations in the proliferative activity of keratinocytes.

Overall, *in vitro* cholesteatoma models have quite a bit of variation in their experimental design. The origin of cells, culture media, glutamine and antibiotics supplementation, as well as cell culture platforms differ substantially between the published studies. As the articles usually do not report success rates and no single model protocol is able to fully duplicate the cholesteatoma pathology, the choice between various *in vitro* cholesteatoma models depends heavily on the intended eventual purpose of the study design.

2.7   Biological tissue adhesives

Biological sealants have made achieving hemostasis during surgery easier, while also reducing often devastating postoperative complications in thoracic and gastric surgery, for example (Bachet and Guilmet 1999, Lee et al. 2004). Fibrin glue imitates the final stages of blood coagulation: human fibrinogen is activated by bovine thrombin and calcium chloride, resulting in a clot that provides adhesive and hemostatic control to the surgeon. During the healing phase, this clot is completely absorbed without foreign body reaction (Brennan 1991). In general, fibrin sealants are mechanically flexible but lack strong adhesion (Annabi et al. 2015). As allogenous products, commercial fibrin glues hold a risk of allergic reactions and

viral transmission, albeit these risks are very low (Yoo et al. 2008). To address the aforementioned safety concerns, equipment that allows collection and isolation of autologous tissue adhesives from the patients' own blood, have become available. However, the action is typically delayed and the adhesion is weaker compared to allogenous, homologous fibrin products (Yoo et al. 2008).

In orthopedic surgery, the increased need for biological adhesives in the repair of musculoskeletal or chondral fractures, for example, has been acknowledged (Shah and Meislin 2013). In ENT or related disciplines, the current official or published off—label application range of fibrin glue includes the benefit of reducing hematoma rates in face-lift surgery (Zoumalan and Rizk 2008) and management of auricular hematomas while at first surgically evacuating the hematoma between the skin and the cartilage (Mohamad et al. 2014). In addition, fibrin glue serves as a safe tool in dural repair (Epstein 2010), an effective fixation method of mucosal flaps in septal surgery (Daneshrad et al. 2003), a possible fixation method in underlay-technique-myringoplasty (Yuasa and Yuasa 2008) and a method to substantially reduce the drain output and the hospitalization period after a selective neck dissection, as the fibrin glue is applied immediately before the skin wound closure (Mushi et al. 2015). Fibrin glue has also been used in order to reduce postoperative drainage after thyroidectomy, parathyroidectomy, parotidectomy, tonsillectomy and adenoidectomy (Yoo et al. 2008).

Katzke and colleagues studied the safety of fibrin glue specifically in regards to ear with their rabbit model, and found the adhesive well tolerated without any toxic effect on the middle or inner ear (Katzke et al. 1983). As far as the treatment of chronic otitis media is concerned via mastoidectomy, it is hard to see an advantage in using fibrin glue alone in mastoid cavity. However, as tissue adhesives generally make surgical procedures easier in one stage or the other, the question of whether biological tissue glues offer possible benefits in mastoid obliteration surgery is intriguing. In their work, Reck and Bernal-Sprekelsen used a rabbit model with femoral bone defects in order to study the effect of fibrin glue on the osseus integration of tricalcium phosphate granules. As a result, they found that fibrin glue impedes osseus integration of TCP for at least 6 weeks, by way of inducing soft tissue development (Reck and Bernal-Sprekelsen 1989). Zazgyva et al. recently studied the effect of fibrin glue on ossification from a different perspective, also with a rabbit model with femoral bone defects. Five weeks after implantation, fibrin glue as a sole defect filler in trochlear osteochondral defects induced fibrosis and cartilaginous tissue, whereas a combination of BG S53P4 granules and fibrin glue led to early signs of bone repair (Zazgyva et al. 2015). In a clinical study, Franco-Vidal and colleagues have published their mastoid cavity obliteration results with ceramic biomaterial consisting 60% of HA and 40% of β-TCP, used in tandem with fibrin glue, and found satisfactory osteointegration and ear canal skin tolerance of this composite material (Franco-Vidal et al. 2014).

Based on the complex nature of fibrin as a biopolymer, one could see the future development of fibrin glue usage orienting towards biological scaffolds in tissue engineering, forming a basis for stem cells to regenerate different tissues (Ahmed et al 2008). Also, the growth factors already found in fibrin glue, combined with added proteins such as fibroblast growth factor (Kanemaru et al. 2011), opens possibilities for new specific tissue engineering therapies that utilize medical composite structures *in vivo*.

2.8 Synthetic tissue adhesives

Among synthetic tissue adhesives, cyanoacrylate derivatives have been used for several decades (Kamer and Joseph 1989). Through very rapid exothermic reaction, where cyanoacrylate monomers attach forming long polymers in the presence of liquid or air humidity, cyanoacrylate tissue adhesives form rigid bonds and offer tight adhesion with high stiffness (Schneider 2009, Annabi et al. 2015). The traditional indication for cyanoacrylate glues has been the treatment for skin wounds (Yoo et al. 2008, Schneider 2009). With time, due to the cyanoacrylates' speed, ease of use (Brown et al. 1996) and substantial mechanical strength of the adhesion (Ahn et al. 1997, García Páez et al. 2004), several other uses have emerged either as official indications, or as more than one hundred off—label applications (Hallock 2001). Currently, one might consider using cyanoacrylate glues in tympanoplasty for graft material fixation (Tuzuner et al. 2015), in the management of central corneal perforations (Jhanji et al. 2011), helping with facial bone fracture adhesion and for controlling the bleeding of esophageal varices and gastric ulcers (Schneider 2009).

The use of short-chain cyanoacrylates, for example ethyl-cyanoacrylate, leads to severe histotoxicity with acute inflammation, tissue necrosis and chronic foreign body reaction. On the other hand, longer-chain cyanoacrylate butyl-2 –cyanoacrylate, has minimal histotoxic effect when used in bone and cartilage graft fixation (Kamer and Joseph 1989, Toriumi et al. 1990). However, it is less suited for subcutaneous use when there is glue contact with well-vascularized soft tissues (Toriumi et al. 1991).

Polyethylene glycol (PEG) –based synthetic sealants have been widely used for sealing suture lines in vascular grafts and to stop CSF leakage following neurosurgery (Wang et al. 2011, Annabi et al. 2014), as well as for decreasing air leakage following lung surgery (Lequaglie et al. 2012). The latest effort in sealant research has been the development of mussel-inspired tissue adhesives, with the goal of acquiring strong adhesion in wet environments as well as good biocompatibility and suitable biodegradability (Annabi et al. 2014). In addition, mimicking the nanotopography of porcupine quills tips, for example, has paved the way for new forms of tissue adhesive patches with strong adhesion, without the possible toxicity issues of chemical tissue adhesives (Annabi et al. 2014).

3   AIMS OF THE STUDY

Aims of the present study were:

1.   To study clinical results of mastoid obliteration surgery using BG S53P4 granules as a mastoid cavity filler.

2.   To evaluate whether fibrin glue has an effect on the solubility of BG granules, and whether this possible effect might have a negative influence on the properties of BG.

3.   To study the mechanical strength of tissue glue – BG S53P4 granule compound and whether cyanoacrylate glue could be used with BG from a mechanical point of view.

4.   To test a hypothesis of cholesteatoma growth –inhibiting properties of BG S53P4 with a suitable *in vitro* keratinocyte model.

4 MATERIALS AND METHODS

4.1 Materials (I–IV)

4.1.1 Patients (I)

After approval of the joint Institutional Committee on Human Research at the University of Turku, a total of 26 patients were included in this clinical retrospective study. 16 patients had chronic otitis media with cholesteatoma, while 9 patients had chronic otitis media without cholesteatoma. Finally, one patient did not have a chronic ear infection, but instead an episode of meningitis had revealed an atraumatic, spontaneus cerebrospinal fluid (CSF) leakage in the region of the middle temporal fossa / mastoid cavity, indicating mastoid cavity obliteration surgery. Three patients had a loss of dural support in caudal direction of the mastoid cavity, owing to the bony dehiscence at the middle cranial fossa. Three patients also had a dural fistula with CSF leakage.

There were 12 male patients and 14 female patients. At the time of the surgery, the median age of the patients was 50 years, the youngest patient being 24 and the oldest 81 years old. Six patients underwent primary surgery, while 20 patients had revision surgery. Audiometric evaluation was performed for each patient before and after surgery, and normal clinical ear inspection was carried out under a microscope. CT scans were performed before surgery, and either CT or MR imaging was utilized also after surgery in selected cases when indicated.

4.1.2 BG granules (I–IV)

The BG S53P4 granules, produced by BonAlive Biomaterials Ltd., Turku, Finland, have been approved for clinical use with appropriate European Conformity CE-mark and US Food and Drug Administration's 510k clearance. The granules were between 0.5 and 0.8 mm in size, and the composition of the granules, as well as that of a comparative commercial product, is listed in Table 4.

Table 4. Composition of two commercial bioactive glasses.

Composition (weight%)	BonAlive® S53P4	45S5 Bioglass®
SiO_2	53	45
P_2O_5	4	6
CaO	20	24.5
Na_2O	23	24.5

4.1.3 Fibrin glue (I–III)

Tisseel Duo Quick (Baxter AG, Vienna, Austria) was chosen as the fibrin glue for this study. It is a two-component sealant and adhesive made primarily of pooled human plasma. The sealer protein solution contains human fibrinogen with fibrinolysis-delaying synthetic aprotinin, while thrombin solution contains thrombin.

The product is offered in several packaging sizes and for this study, 2 ml versions were selected. It is stored in a freezer and becomes ready to use after the frozen pre-filled syringes are warmed to a temperature between 33 and 37 °C with the help of a water bath or an incubator. When warmed and unopened, fibrin glue can be used for 48 hours. In practice, after each application of the glue there is a need to change the application cannula due to coagulation of the glue inside the cannula. The formed seal achieves 70 percent of its maximum strength in 10 minutes, and after 2 hours its full mechanical strength is achieved.

4.1.4 N-butyl-2 cyanoacrylate tissue adhesive (III)

Histoacryl® (B. Braun Surgical SA, Rubi, Spain), n-butyl-2-cyanoacrylate (CA) – tissue adhesive, is a totally synthetic monomer system. The 0.5 ml ampoules are stored at room temperature. There are two color versions, blue and translucent. For this study, the undyed, translucent version was chosen. When the opened product has contact with any surface containing water, it polymerizes exothermically. In other words, monomers bond into long chains and to the underlying surface. This reaction takes place within seconds and also produces heat. Skin wound closure and hernia mesh fixation are approved uses for Histoacryl®. Nevertheless, the manufacturer discloses other off-label applications as well.

4.1.5 Immersion media (II, III)

When qualities of BG-fibrin glue – and BG-n-butyl-2-cyanoacrylate glue – composites were studied in regards to pH and weight change, simulated body fluid (SBF) was used as an immersion medium. SBF, prepared according to the detailed Kokubo protocol (Kokubo and Takadama 2006), offers a valuable tool in bioactive material research. *In vivo* apatite formation on the surface of a bioactive material can be reproduced in SBF (Kokubo and Takadama 2006), thus bone bioactivity of a material can be predicted by observing surface apatite formation in SBF *in vitro*.

SBF is manufactured by dissolving 10 different reagents into 36.5 °C distilled water, starting with NaCl, each reagent being introduced into the solution only when the previous reagent has already dissolved completely. In the end, the pH of the solution is adjusted to 7.40 at 36.5 °C. Ion concentrations of SBF are very similar to those of human blood plasma.

Purified water was used as an immersion medium for compression strength and water sorption measurements.

4.1.6   *HaCaT keratinocytes and cell culture media (IV)*

Immortalized human skin keratinocytes were chosen as an *in vitro* –cholesteatoma model. These HaCaT keratinocytes were provided by BioCity Turku (University of Turku, Finland). The initial cell culture took place in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, United States). DMEM was supplemented with 10 % fetal bovine serum, 1% minimum essential medium non-essential amino acids (MEM-NEAA, Thermo Fisher Scientific, Waltham, MA, United States) and 0.5 % penicillin/streptomycin solution (PenStrep, Lonza Group Ltd., Basel, Switzerland). Besides DMEM, adult keratinocyte growth medium (KGM, Sigma-Aldrich, St. Louis, MO, United States) was also used in the HaCaT experiments. KGM was only supplemented with 0.5 % PenStrep, as opposed to DMEM.

4.2   **Methods (I–IV)**

4.2.1   *Surgical technique (I)*

All patients were operated on under general anesthesia. To facilitate hemostasis, local 1 % lidocaine-epinephrine -solution was injected subcutaneously, and a retroauricular skin incision was made to gain a wide exposure and visualization. Temporal fascia graft was harvested, and a meatally based Palva musculoperiosteal flap was raised using a raspatory, exposing either the intact mastoidal bone surface or a previous mastoidectomy cavity. A CWD mastoidectomy followed on the patients not previously operated on, excluding four patients, with whom CWU mastoidectomy was determined to provide sufficient exposure. A meticulous revision surgery was undertaken on previously operated patients.

Mastoidal air cells were carefully opened with a burr and all mucous membranes were meticulously removed, along with middle ear cholesteatoma when present. The drilling process proceeded until a dense, normal bone surface was encountered and until sinus sigmoideus, base of middle cranial fossa and sinodural angle were all polished. When indicated, ossiculoplasty was undertaken using a modified remnant of a suitable ossicular bone as a columella between the tympanic membrane and plate of stapes / oval window, or an artificial prosthesis when applicable. Myringoplasty was carried out using temporalis muscle fascia with tragal or conchal cartilage chips, utilizing an inlay graft technique. In such a case, pieces of Spongostan Special (Ethicon Inc., NJ, USA) were used in the tympanum to provide sufficient support medial to the fascia graft.

In the latter part of the operation, a thoroughly cleaned mastoidal cavity was filled with BG S53P4 granules, additional bone paté with 8 patients requiring a small additional obliteration material volume, and fibrin glue. The BG granules were moistened with physiological saline solution, and the required volume of BG granules varied from patient to patient from two to 20 cm^3. In CWD patients, careful attention was paid to cover BG granules in the reconstructed posterior meatal wall area with temporalis muscle fascia and Lyoplant® (B. Braun, Melsungen, Germany). Palva musculoperiosteal flap was used in the retroauricular area to cover the obliterated mastoid cavity, respectively. A meatoplasty was performed for 19 patients.

Three patients had a bony dehiscence at the middle cranial fossa and a following loss of dural support. For these patients, support in caudal direction and an anatomically correct position of the dura were achieved using a BG plate, formerly applied in blow-out fractures of orbital floor (Peltola et al. 2008). Three patients had dural fistulae with CSF leakage as a result, and one of these patients suffered from a perioperative iatrogenic fistula. These patients were treated using CWU mastoidectomy route: occlusion of the fistula with a fascia graft and Lyoplant® patch, with fibrin glue attachment, in addition to mastoid cavity BG obliteration, were performed.
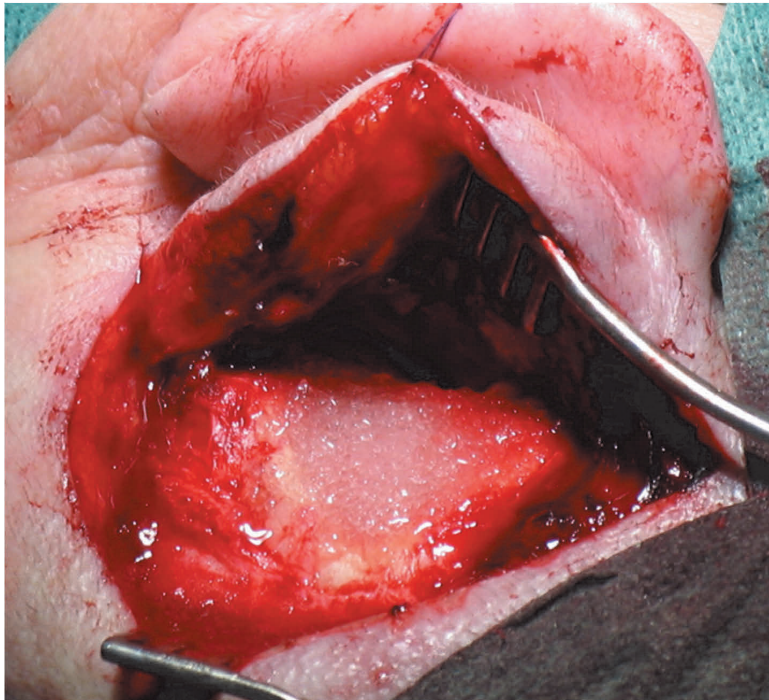
Figure 4. Mastoid cavity filled with bioactive glass granules. Photo: Jaakko Pulkkinen.

Finally, nylon sutures (Ethilon®, Ethicon Inc., NJ, United States) were used to close the retroauricular skin incision. The ear canal was filled with wicks (Merocel®, Medtronic Inc., CT, United States) and gauze. These materials, as well as skin sutures, were removed typically one week after surgery. In the postoperative period, patients received pain medication. Postoperative antibiotic treatment was prescribed for 13 patients.

4.2.2   Absorption test (II)

Polyvinyl siloxane molds (Coltène® Lab-Putty, Coltène/Whaledent AG, Altstätten, Switzerland), with a cylindrical hole of 5.0 mm in diameter and 10 mm in depth, were filled with 0.14 g of physiological saline solution –moistened BG granules. A drop of fibrin glue, having a temperature of either 9, 21 or 37º C, was applied on top of each granule layer. After 24 hours at 21 °C room temperature, the mold and all loose particles from the solid BG-fibrin glue –piece were removed, and the maximum thickness of the solid piece, reflecting the penetration depth of fibrin glue into the interparticle space of BG particles, was measured using light microscopy.

4.2.3   pH test (II)

The effect of fibrin glue on pH values in a liquid environment was studied using simulated body fluid (SBF), prepared according to the Kokubo protocol (Kokubo and Takadama 2006). After 0.14 g (SD 0.00) of BG S53P4 particles were weighed (XS105 Dur –laboratory scale, Mettler Toledo, United States) for each six test tubes, 35 ml of SBF was added. A seventh test tube, containing only SBF without BG, served as a control. Test tubes were kept at 37 °C in a shaking incubator (Grant OLS200, Grant Instruments, United Kingdom) with a 100 rpm shaking frequency, and SBF pH values were measured (PHM220 Lab pH Meter, Radiometer, Copenhagen, Denmark) at 21 °C room temperature after 1, 2, 3, 4 and 7 days. In this first test series, BG was tested in SBF by itself, without fibrin glue.

In a second test series, a similar amount of BG S53P4 particles and SBF were used. In addition, 0.13 g (SD 0.04) of fibrin glue was added. Otherwise pH measurement protocol followed previous description, and as an additional control, two samples of fibrin glue, without BG, were incubated in SBF to observe the potential pH effects of mere fibrin glue.

4.2.4   Ion dissolution test (II)

Twenty-two BG-fibrin glue –samples were manufactured as described in 4.2.2, with mold dimensions of 8 mm by 6 mm (diameter and height, respectively) and sample composition of 0.25 g of BG S53P4 particles (SD 0.00) and 0.18 g of fibrin glue (SD 0.04). The samples solidified for 18 hours at 21 °C room temperature

under a 0.125mm thick Mylar® polyester film. Samples were then immersed in 10 ml of SBF and kept in a shaking incubator for up to 14 days.

During SBF immersion, concentration of calcium, potassium, magnesium, sodium, phosphorus and silicon elements were measured after 2, 5, 9 and 14 days with inductively coupled plasma optical emission spectrometry (ICP-OES, Perki-nElmer Optima 5300 DV, United States). In addition, combined weights of the solid sample and any detached BG particles from SBF were measured to determine weight loss. SBF pH values (37 °C) were measured at 0, 1, 2, 4, 5, 6, 7, 8, 12 and 14 days.

4.2.5   Compression strength and water sorption of BG-CA –composites (III)

To investigate whether CA glue is a viable alternative in BG granule fixation from a mechanical standpoint, a compression strength test was planned based on the ISO 4104 –standard. Polyvinyl siloxane moulds (Coltène® Lab-Putty, Coltène/Whaledent AG, Altstätten, Switzerland) were used to produce cylindrical BG particle-CA glue –composites with a diameter of 4.0 mm and a height of 6.0 mm. After the molds were filled with BG particles, CA glue was applied on top of the particle layer. Due to the capillary phenomenon, glue rapidly spread into the interparticle space. After the quick polymerization process was complete, molds were removed and composite sample ends were treated into parallel end surfaces.

BG-CA –composites' strength after water immersion was tested as follows: eight specimens per group were made for the dry group, 3-day immersion group, 10-day immersion group and the 30-day immersion group. After the correct water immer-sion time was reached, by keeping the specimens covered by a thoroughly moist cotton wool at 37°, the compression strength tests were performed with Lloyd LR30K+ (Lloyd Instruments, Ametek Inc., West Sussex, United Kingdom) uni-versal material testing device, with a 2500 N load cell size. While each cylindrical composite was attached firmly to the attachment jaws, a velocity of 1 mm per mi-nute was used to compress the sample. Nexygen plus –software (Lloyd Instru-ments, United Kingdom) was used to record stress at maximum load, strain at max-imum load and Young's modulus in compression. In addition, the specimens were weighed at the beginning of the experiment as well as after the correct period of water immersion, using a Mettler Toledo PB303-S –laboratory scale (Mettler To-ledo Inc., Columbus, OH, United States).

4.2.6   Dissolution of BG-CA– and BG-fibrin glue –composites (III)

BG-CA glue –composites, BG-fibrin glue –composites and BG particles were in-cubated in SBF and pH change was measured using a PHM220 Lab pH Meter (Radiometer, Co-penhagen, Denmark). BG-CA –composites consisted of 0.125 g

of BG and 0.125 g of CA glue, BG-fibrin glue –composites consisted of 0.125 g of each material and finally in the control group, 0.125 g of BG particles was used. Six specimens in each group were incubated in 10 ml of SBF at 37°C for 14 days, while SBF pH was measured at 1, 2, 3, 4, 7 and 14 days' incubation time points. In addition, samples were briefly removed from SBF for weighing in order to observe the weight loss.

4.2.7   *HaCaT cell viability test (IV)*

After thawing the kryopreserved HaCaT cells, keratinocytes were incubated in 5 % CO_2 atmosphere at 37 °C in cell culture flasks. The supplemented DMEM medium was replaced every 2 days. When the keratinocytes reached 80–90 % confluence, confirmed visually by light microscopy (Nikon Eclipse TS100, Nikon Corporation, Tokyo, Japan), the cells were separated from the flasks with 0.25 % trypsin-EDTA-solution and re-cultured. HaCaT cells were used for experiments after undergoing 5–7 passages.

Two types of cell culture media were prepared for the HaCaT cell viability test. At first, BG S53P4 granules were weighed (Mettler Toledo AB-104S/Fact, Columbus, OH, United States) into 15 ml Falcon tubes. An exact volume of culture media, either supplemented DMEM or KGM, was then added into the tubes so that concentrations of 0.5 %, 1 %, 2.5 %, 5 % and 10 weight-% of BG were acquired in both media groups. In order to promote BG granule solubility in medium without possibly degrading the active protein content of the media, BG-DMEM and BG-KGM-solutions were incubated at 4 °C temperature for 5 days in a WMR rotating mixer. At 24 hours before use, the solutions were relocated to 37 °C. Finally before usage in HaCaT cultures, the BG-DMEM and BG-KGM-media went through a pH analysis (Mettler Toledo Mini MR1basic pH meter, Columbus, OH, United States).

For the purpose of the cell viability test, keratinocytes were seeded in 96-well plates. The cell density for this experiment was 161 000 cells/mL, measured with a Bio-Rad TC20 automated cell counter (Hercules, CA, United States). DMEM with a volume of 0.1 ml was used initially in each well. Incubation took place in 5 % CO2 atmosphere at 37 °C, and after confirming with a light microscope an average cell confluence of ≥90 % after 48 hours of incubation, the cell viability experiment was initiated. The original medium of each well was replaced with a test medium, with a volume 0.1 ml per well. Upon completion, 24 wells served as a control in both DMEM and KGM groups, and in comparable fashion the test media groups with BG concentrations of 0.5 %, 1 %, 2.5 %, 5 % and 10 % also had 24 wells for each concentration, with a total amount of 288 experimental wells.

HaCaT cell viability was determined with the MTT method (Promega corporation, Madison, WI, United States), in a phase when an incubation period of 48 hours was reached for the 96-well plates. In this method, cell viability is concluded by the magnitude that tetrazolium dye is being reduced to its insoluble form by viable cells, with accompanying color change of the solution. As only the viable cells are able to change the color of the MTT dye, a numerical value of cell viability can be obtained with a light spectrometry measurement. In practice, 15 µL of MTT solution was first added into each well, followed by the addition of 100 µL of MTT solvent two hours later. Within one hour of additional incubation following the manufacturer's instructions, light absorbance was measured with a wavelength of 570 nm (Ensight Multimode Plate Reader, PerkinElmer, Waltham, MA, United States). In order to obtain a base light absorbance level, also blank controls of DMEM, KGM and empty wells were measured.

4.2.8   *HaCaT scratch test (IV)*

The proliferative capability of HaCaT keratinocytes in the presence of BG was tested with a scratch assay experiment. HaCaT cells were incubated in 24-well plates using a density of 59 900 cells/mL for 48 hours. At this stage the cultures were confluent, and all of the media, 0.4 ml of DMEM in each well, was removed. Thereafter, the bottom of each dry well was scraped with a pipette tip, approximately 0.5 mm in diameter, in order to produce a vertical scratch wound into the monolayer of keratinocytes. Fresh DMEM of 0.4 ml per well was added. A control group consisted of six wells without BG S53P4 granules, whereas in the experimental groups there were 0.9 %, 1.7 % and 3.5 % of added BG S53P4 granules by volume, respectively. Hence, in the experimental groups the BG granules were in contact with the confluent, excluding the scratch area, keratinocyte cell layer. The incubation took place in 5 % CO_2 atmosphere at 37 °C. The alterations in cell confluency and morphology were documented with light microscopy and –photography (Nikon Eclipse TS100, Nikon Corporation, Tokyo, Japan) in the beginning of the experiment, and after an incubation period of 24 and 48 hours.

4.2.9   *HaCaT morphology and cytokine profile of the culture medium (IV)*

The possible morphological changes that BG S53P4 granules might induce to HaCaT cells were studied by first seeding keratinocytes in 24-well plates at a cell density of 123 000/mL. Similarly to the scratch test, 0.4 ml of DMEM was used in each well with an incubation period of 48 hours. The wells with 100 % confluency were chosen for experiments. After DMEM replacement, BG S53P4 granules were added into each well in the experimental group, whereas control wells contained only fresh culture medium. After an additional incubation period of 48 hours, the medium was carefully removed from each well, with an endeavor to cause as little disturbance to the well plate as possible. Six samples from 0 % BG-wells and 6

samples from 2.5 % BG- wells underwent analysis for interleukine-6 (IL-6) and interleukine-8 (IL-8) concentrations, using Merck Millipore Human Cyto-kine/Chemokine Magnetic Bead Panel (Merck, Darmstadt, Germany). The dry cell cultures were then immediately studied and photographed with light microscopy.

4.2.10 Statistical analysis

For the second study, SPSS Statistics software (IBM Corporation, NY, United States) was used in statistical analysis. SBF pH change during incubation in both BG and BG-fibrin glue –groups, as well as between these two groups, was compared with repeated measures analysis of variance (rm ANOVA). The statistical significance was set at the $p < 0.05$ level.

For the third study, analyses were performed with SAS System (version 9.3 for Windows) or SAS JMP (version 11.1.1). When data was normally distributed, one-way analysis of variance was used for comparing strength, Young´s modulus and water sorption of the BG-CA –composites. When data was not normally distributed, the Kruskal-Wallis test was chosen. A hierarchical linear mixed model was used in the analysis of mean pH changes of SBF. Again with all methods, p-values less than 0.05 were considered as statistically significant (two-tailed).

For the fourth study, SAS JMP (version 11.1.1) was used in statistical analysis. One-way analysis of variance was used to compare different specimen groups with varying concentrations of BG. In the scratch assay test the data was not normally distributed, and Kruskal-Wallis test was used for comparisons between the groups. P-values less than 0.05 were considered statistically significant.

5   RESULTS

5.1   Clinical study (I)

The average duration of hospitalization after the surgery varied between 2 and 11 days, with a 5.3 day-average. More recent surgery favored shorter hospitalization periods. The goal of reducing the size of the cavity was achieved with 92 % of the patients, while 27 % of the patients had even a nearly normal appearance of the posterior meatal wall and hence a completely eliminated radical cavity. In two patients with either a widely infiltrating cholesteatoma or dural fistula, the ear canal was completely closed as part of a petrosectomy.

The mean follow-up period was 42.5 months (range 1–182 months). The reasons for the shortest follow-up periods were either very recent surgery or patients being lost to follow-up. One patient died of an unrelated cause two months after the mastoid obliteration surgery. After surgery of the 26 patients, 18 patients had a well-ventilated middle ear, while 5 patients were seen with a retracted tympanic membrane. One patient developed ultimately a tympanic membrane perforation. Twenty-one patients had dry ears after the surgery, while two patients with occasional otorrhea were treated with debridement and antibiotics, as required, on an outpatient basis. The three patients with the shortest follow-up periods of 1 to 3 months were still seen with moist, small cavities, where the process of epithelialization was incomplete.

Because the majority of the patients had sensorineural hearing loss, hearing restoration was not considered achievable. Only six patients with notable conductive hearing decline were treated by means of ossiculoplasty, and the average result from this additional effort was a 4.0 dB hearing gain. When both preoperative and postoperative hearing evaluation was available, the mean pure tone average (PTA) for the operated ear was 51.9 dB preoperatively and 53.6 dB postoperatively, when three preoperatively essentially deaf ears were excluded.

BG S53P4 granules and three BG plates for skull base support were well tolerated, with no adverse effects. Two patients had a protrusion of a small amount of BG granules in their ear canal, owing to evidently inadequate fascia coverage of the granules. With one patient this resulted in delayed healing with several additional outpatient visits, while the other patient underwent re-operation.

Table 5. Patients in the clinical study. Adapted with modifications from Study I.

Pt	Sex	Age at surgery (y)	No. of previous operations	Surgical treatment	Pure tone average (dB) PreOp/PostOp	Follow-up (mo.)	Postoperative outcome	Complications
1.	F	50	1	MO with BG, OCR	70 / 76,25	71	dry, small cavity	no
2.	M	41	none	MO with BG, OCR	40 / 36,25	182	dry ear, normal ear canal	no
3.	M	35	1	MO with BG	11,25 / 5	94	dry, medium-sized cavity	no
4.	F	63	1	MO with BG, OCR	76,25 / 71,25	116	dry, small cavity	no
5.	M	30	3	MO with BG	38,75 / not known	111	dry, medium-sized cavity	no
6.	F	70	3	MO with BG	93,75 / not known	93	dry, small cavity	prolonged healing
7.	M	50	2	MO with BG, OCR	50 / 20	52	dry ear, normal ear canal	no
8.	F	81	none	MO + skull base support with BG	77 / deaf	37	dry ear, closed ear canal	preop. partial, postop. total facial paresis
9.	F	56	2	MO with BG	52,5 / 66,25	55	dry ear, large cavity	no
10.	M	72	none	MO with BG + ODF	/ 50	24	dry ear, normal ear canal	no
11.	F	77	1	MO with BG + ODF	28 / not known *	2 *	dry ear, normal ear canal	no
12.	F	47	none	MO with BG	17,5 / 17,5	55	intermittent otorr-hea, normal ear	postoperative vertigo & pain
13.	M	43	4	MO + skull base support with BG	80 / 80	36	dry ear, closed ear canal	no
14.	M	31	2	MO with BG	42,5 / not known	46	dry ear, medium sized cavity	no
15.	M	41	1	MO with BG	not known	1	moist, small cavity	no
16.	M	31	4	MO with BG	deaf	33	dry, small cavity	BG granules exposed, prolonged healing
17.	F	48	1	MO with BG	27,5 / 26,25	36	dry, small cavity	no
18.	M	52	5	MO with BG	deaf	3	moist, medium sized cavity	wound opened post-operatively, re-operation
19.	F	62	2	MO with BG	38,75 / 45	3	dry, small cavity	no
20.	F	33	4	MO with BG	21,25 / not known	21	intermittent otorr-hea, large cavity	no
21.	M	62	1	MO with BG, OCR	70 / 67,5	11	dry, small cavity	no
22.	F	63	1	MO with BG	73,75 / 75	6	dry ear, normal ear canal	no
23.	F	24	1	MO with BG	63,75 / 66,25	6	dry, small cavity	no
24.	F	32	none	MO with BG + ODF	2,5 / 8,75	7	dry ear, normal ear canal	intraoperative dural lesion
25.	F	75	none	skull base support with BG plate + OCR	46,25 / 57,5	4	dry ear, normal ear canal	no
26.	M	78	1	MO with BG	deaf	1	moist, small cavity	no

BG=bioactive glass; MO=mastoid obliterarion; OCR=ossicular chain reconstruction; ODF=occlusion of dural fistulas

* died after 2 months follow-up

5.2 In vitro –material study: solubility and mechanical properties of tested biomaterials (II)

Penetration depths of fibrin glue within BG particles, as demonstrated in Figure 5, were 4.2 and 6.4 mm for 9 °C fibrin glue; 3.2 and 5.6 mm for 21 °C fibrin glue and 3.72 and 4.05 mm for 37 °C fibrin glue.
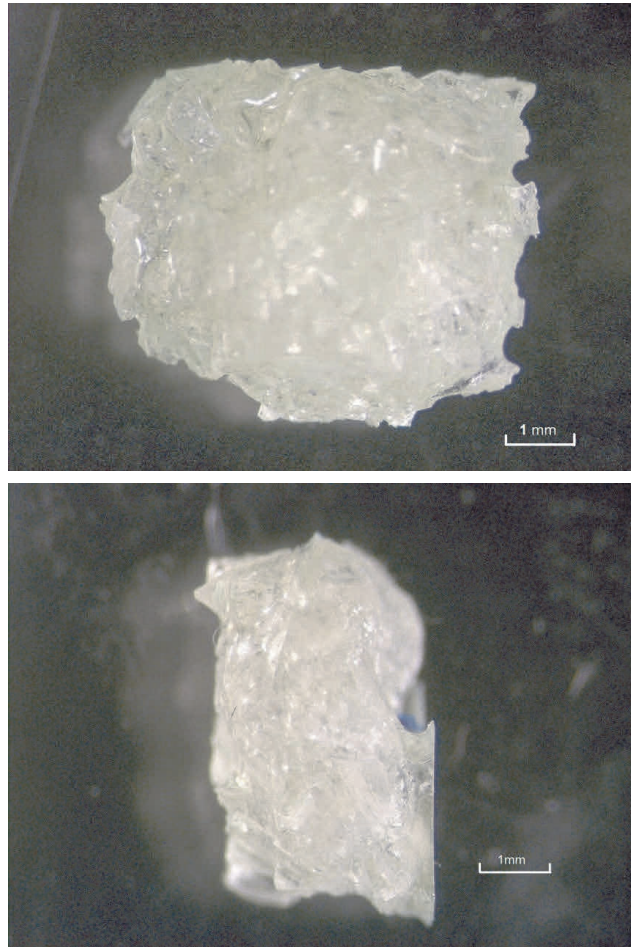

Figure 5. Penetration depth of fibrin glue within BG particles varied between 3.2 and 6.4 mm. Photo: Markus Hiltunen.

In the pH test protocol, incubating solely fibrin glue in SBF did not yield any change in pH, as all the measured pH values stayed between 7.48–7.49 during the whole incubation period. BG S53P4 particles, on the other hand, produced continuous pH increment from the initial average value of 7.6 to 8.0 (SD 0.1), within seven days of incubation (p<0.001).

BG S53P4 particles together with fibrin glue produced a comparable SBF pH increment, as the average pH of SBF increased from 7.5 to 8.0 (SD 0.0) during seven days on incubation. The pH change within this group was statistically significant (p<0.001), and compared to the pH change pattern of BG particles –only group on a daily basis, a statistically significant difference was found (group x time interaction effect, p=0.008).
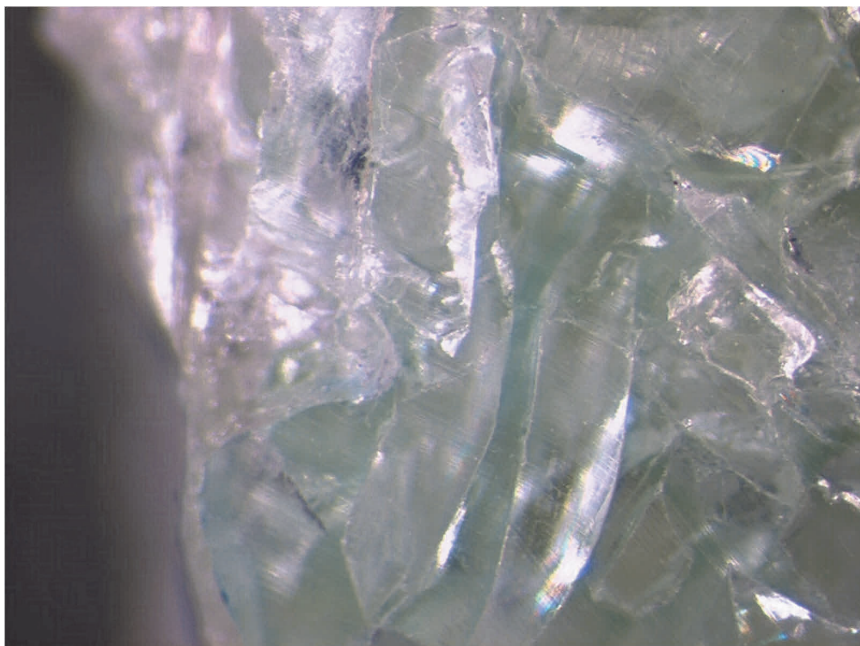
Figure 6. Light microscopy detail of a composite of BG granules and fibrin glue. Photo: Markus Hiltunen.

In a two week test protocol, BG-fibrin glue –combos lost weight rapidly in the first two days of SBF immersion, after-which the weight was stable. In concordance with earlier samples, the average SBF pH value rose to around 8.0 (Figure 7). During the 14-day incubation period of the BG-fibrin glue –combos, several ion concentration changes were seen in SBF, shown in Figure 3. Calcium ion concentration increased over a two-day-period from 68 mg l^{-1} 150 mg l^{-1}, and started to decrease after 5 days. Silicon ion concentration increased up to 9 days, while phosphorus ion concentration decreased to zero due to Ca-P formation on the glass surface. Concentrations of K, Mg and Na ions stayed at a proportionately constant level.
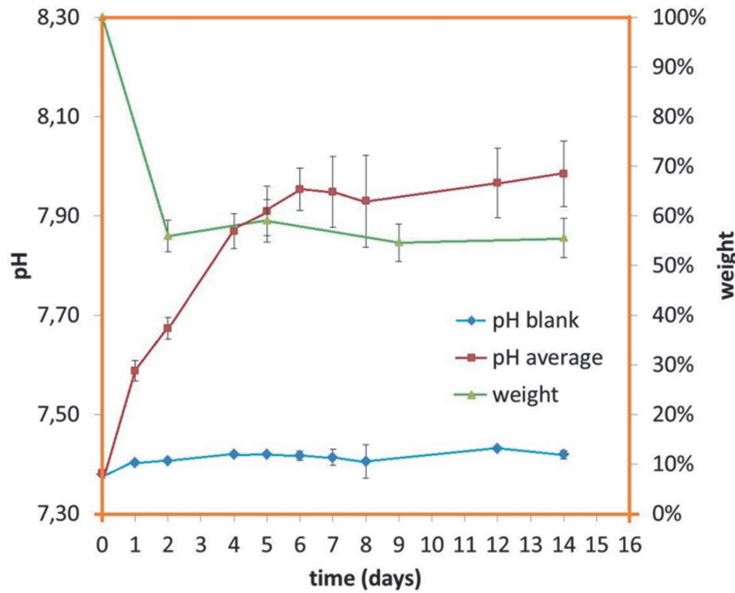
Figure 7. Bioactive glass S53P4 and fibrin glue in simulated body fluid – 14 days incubation with pH monitoring and BG-fibrin glue –particle weight measurement. Reproduced, with permission, from Study II.
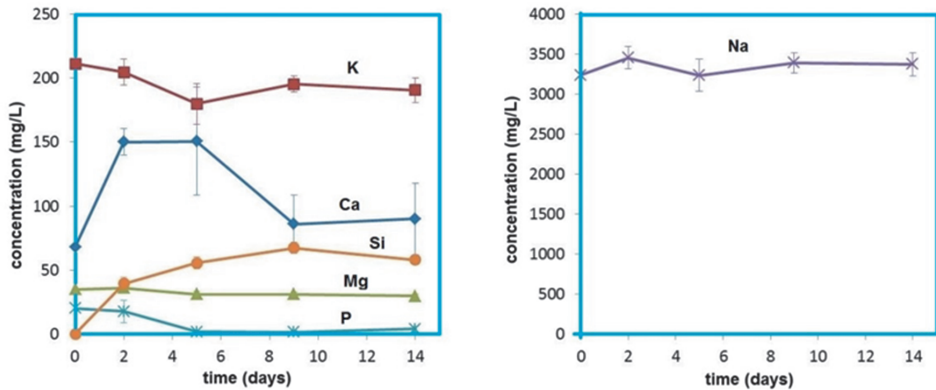
Figure 8. Bioactive glass S53P4 and fibrin glue in simulated body fluid – 14 days incubation with concentration of ions (mg l^{-1}). Reproduced, with permission, from Study II.

5.3 In vitro –material study: solubility and mechanical properties of tested biomaterials (III)

As BG-CA –composites, BG-fibrin glue –composites and BG particles were incubated in SBF, the pH increased in all groups. The average pH after 7 days of incubation increased from 7.35 to 7.94 (SD 0.025) in fibrin glue-BG particle –group, from 7.35 to 7.93 (SD 0.069) in BG particle –group, and from 7.35 to 7.52 (SD 0.066) in the CA-BG –composite group, respectively. A statistically significant pH

change difference was observed between the groups for the whole 14-day period (group x time interaction effect): CA-BG –group versus fibrin glue-BG –group (p<0.001), CA-BG –group versus BG -group (p<0.001) and fibrin glue-BG –group versus BG -group (p=0.0217).

When immersed in water, CA glue-BG particle –composites gained weight, a material behavior opposite to BG granules or fibrin glue-BG –composites, that lost weight during SBF incubation by comparison. After 30 days of water immersion, the mean weight gain of 0.00229 g (p<0.001), or approximately 1.5 %, was observed for CA-BG –composites. There were no statistically significant differences between groups of 3, 10 or 30 days' water immersion (p=0.43), so water sorption did not correlate with immersion time.
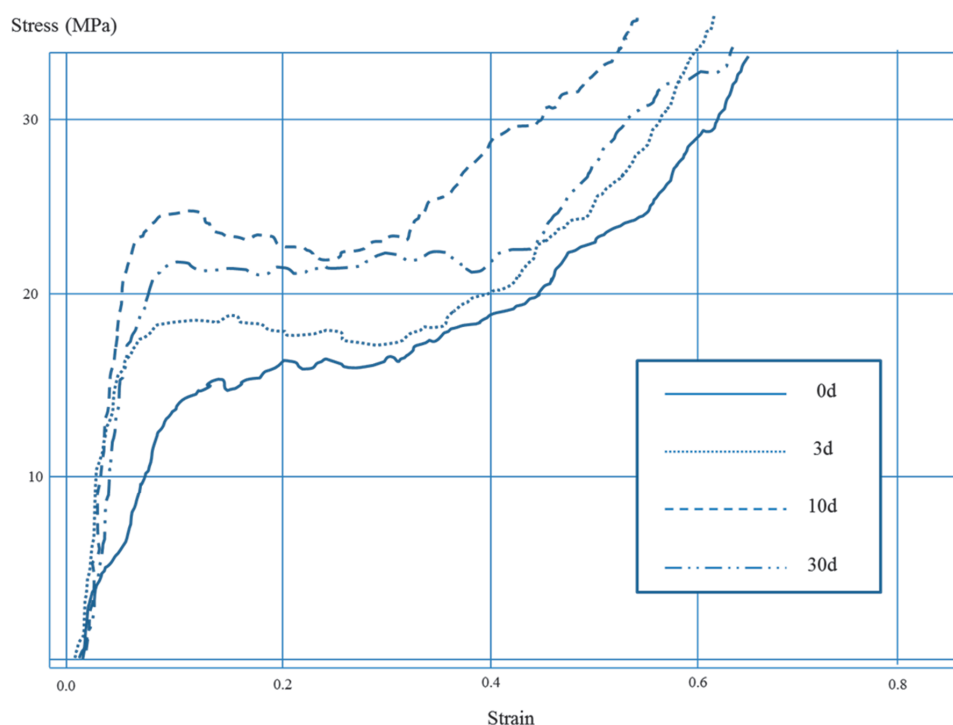
Figure 9. Typical stress-strain curves of the compression load test for CA glue-BG – composites, after 0, 3, 10 or 30 days purified water immersion. Modified from Study III.

Different water immersion periods did not alter compression strength (stress at maximum load) of CA-BG –specimens in a statistically significant manner. For dry samples, the mean stress at maximum load was 35.3 MPa (SD 9.9 MPa). The 3-day immersion yielded a compression strength of 28.9 MPa (SD 9.9, p=0.31), 10-day immersion 28.6 MPa (SD 7.0, p=0.27) and 30-day immersion 36.3 MPa (SD 5.6, p=0.99), respectively. The mean strain at maximum load for dry CA-BG –specimens was 0.66 (SD 0.01), whereas the mean strain for the 3-day immersion

group was 0.59 (SD 0.21, p=0.56 compared to the dry specimen group), 0.47 (SD 0.15, p=0.014) for 10-day immersion group and 0.58 (SD 0.017, p=0.48) for the 30-day immersion group.
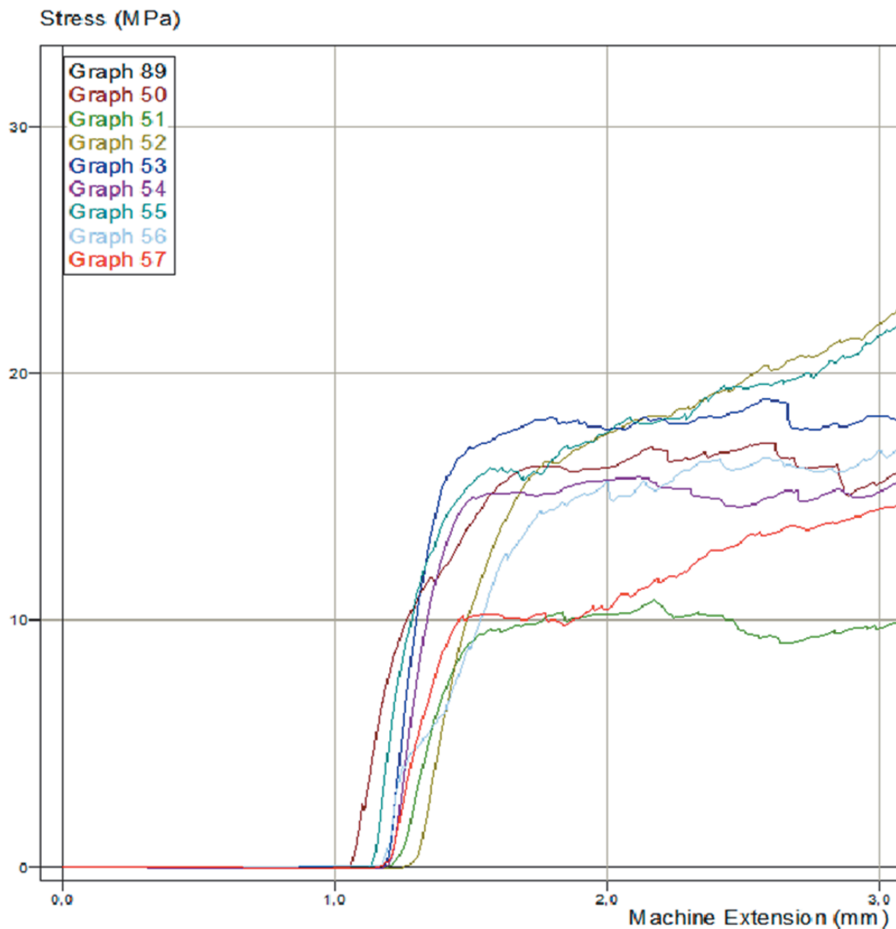
Figure 10. An example of the raw data (Nexygen plus –software, Lloyd Instruments, United Kingdom) from compression strength measurements of CA glue-BG particle – composites, without water immersion.

The dry CA-BG –specimens had a mean Young's modulus of 447.9 MPa (SD 113 MPa). After water immersion for 3, 10 or 30 days, the observed mean values for Young's modulus were 577.5 MPa (SD 102), 616.4 MPa (SD 92.8) and 519.4 (SD 89.7) MPa, respectively. As illustrated in Figure 11, a statistically significant difference was observed between the dry and the 3-day immersion group (p<0.04) and between the dry and the 10-day immersion group (p=0.006). No statistically significant difference was noted, however, between the dry and the 30-day immersion group (p=0.36).
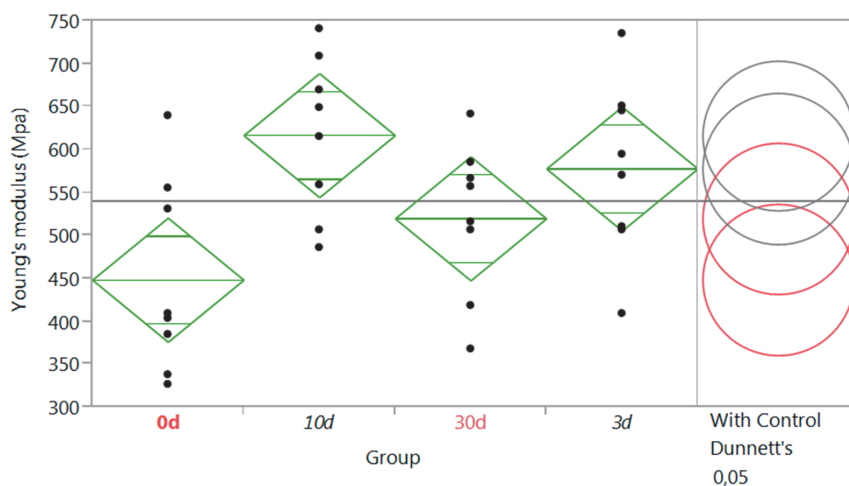
Figure 11. One-way analysis of Young's modulus (MPa), with 95 % confidence intervals shown, for CA-glue-BG –composites after 0, 3, 10 or 30 days of purified water immersion. Reproduced, with permission, from Study III.

5.4   The effect of BG S53P4 on HaCaT keratinocytes (IV)

After an incubation period of six days in a rotating mixer, BG-DMEM and BG-KGM-solutions underwent pH measurements. At 21 °C temperature, the pH of DMEM control was 7.43, whereas in the 0.5 % BG group pH of 7.70 was observed. As the concentration of BG S53P4 granules in the DMEM increased, so did the pH value reaching finally the highest value of 8.43 with a 10 % BG solution. The pH of KGM control solution was 7.35, and the highest observed pH value was 8.58, respectively (Figure 12).

In the MTT cell viability test when several different BG-DMEM and BG-KGM media were used, there was a statistically significant difference in 570 nm light absorbance, when the plain media control groups were compared to the combined BG-groups (Figures 13 and 14). While the mean absorbance was 1.28 (95 % CI = 1.17–1.39) in the 0 % BG-DMEM group, a smaller absorbance value of 1.15 (95 % CI = 1.10–1.19, p=0.0243) was observed for the combined BG-DMEM groups, meaning fewer viable keratinocyte cells in the BG groups compared to the control group (Figure 13). The mean absorbance for the control KGM group was 0.86 (95 % CI = 0.74–0.97), whereas the mean absorbance for combined BG-KGM groups was 0.67 (95 % CI = 0.62–0.72, p= 0.0034), respectively (Figure 14). A regression analysis demonstrated a linear dose-response relationship in the BG-DMEM-group (p= 0.0104, correlation= -0.2086), whereas a statistically significant linear dose-response relationship was not seen in the BG-KGM group (p=0.8202, correlation= -0.0187).

In the scratch assay test, the original pipette tip scratch area in the confluent HaCaT cell layer was considered 0 % confluent. Confluence was estimated after 24 hours

and 48 hours of incubation with microscopy, using also cell culture photographs as a reference. Keratinocyte confluence in the wells without BG, with 0.9 % BG, with 1.7 % BG and with 3.5 % BG were as follows: 0–30 % (after 24 hours) / 10–100% (after 48 hours), 0–20 % / 0–50 %, 0–10% / 0–10 %, and 0 % / 0 %, respectively. Indeed, the 3.5 % BG group showed no HaCaT growth in the area of the scratch at all. Instead, the scratch area had widened and there was a considerable amount of HaCaT cell debris in the culture well. The confluence difference between the 0 % BG control group and BG-groups, analyzed using the Kruskal-Wallis test, was statistically significant (p=0.0031).

The morphology of HaCaT keratinocytes under the influence of BG S53P4 granules was studied in 24-well plates, with a confluent cell culture as a starting point. In the wells without BG or in the BG groups without immediate BG granule contact, an intact monolayer of keratinocytes was seen (Figure 15). In the immediate contact of BG granules, an 8–10 cell thick layer of dead keratinocytes were seen (Figures 16–19). In this dead HaCaT cell region near the BG granules, the outer cell structure was still perceivable, yet there were no nuclei in these cells. Furthermore, an observable transition cell layer with the nuclei and nucleoli still present, was seen between the normal and dead keratinocytes in many wells.

After a two-day incubation period, DMEM samples were obtained for IL-6 and IL-8 evaluation. In the control group without BG granules, mean IL-6 and IL-8 values of 23.0 pg/ml (SD 7.1) and 560 pg/ml (SD 87) were measured, respectively. In medium samples from 2.5 % BG wells, on the other hand, mean IL-6 and IL-8 values of 9.71 (SD 4.9) and 364 (SD 64) were measured. The difference in the IL-6- and IL-8-values between the 0 % and the 2.5 % BG groups was statistically significant (p=0.0038 and p=0.0013, respectively).
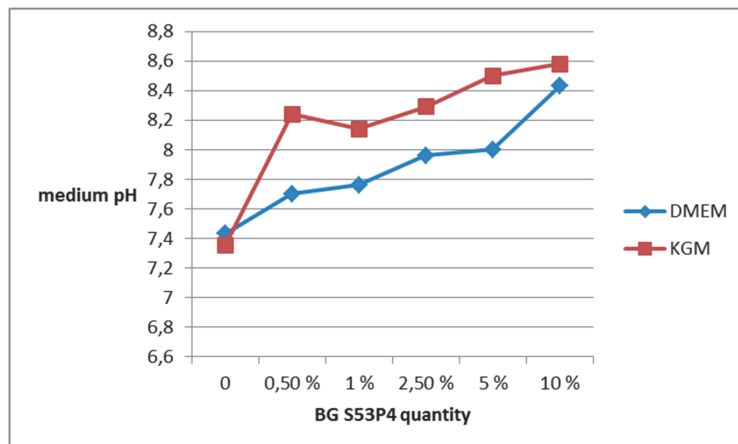
Figure 12. pH levels in Dulbecco's modified Eagle's medium (DMEM) and keratinocyte growth medium (KGM) with different BG S53P4 granule concentrations after a 6-day incubation period. Modified from Study IV.
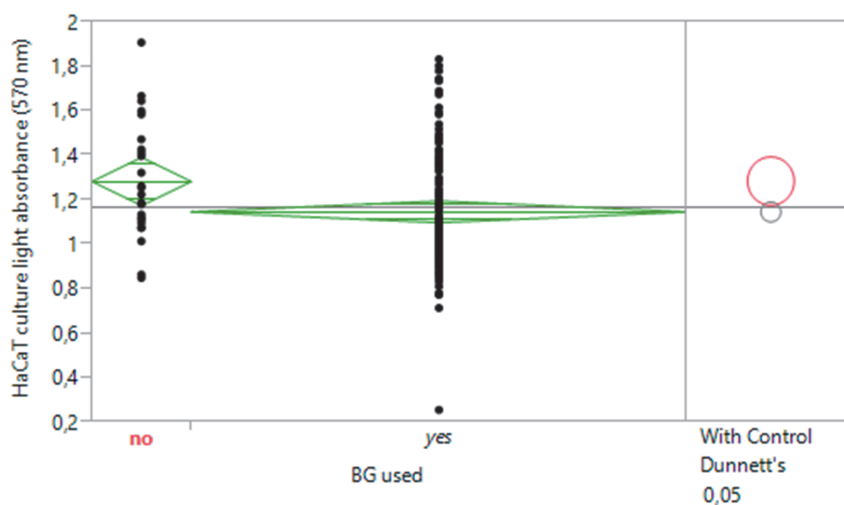
Figure 13. One-way analysis of variance of keratinocyte viability with MTT method. Light absorbance measured with a 570 nm wavelength for cell cultures in DMEM without BG and for cell cultures containing 0.5–10 % BG S53P4 in DMEM.
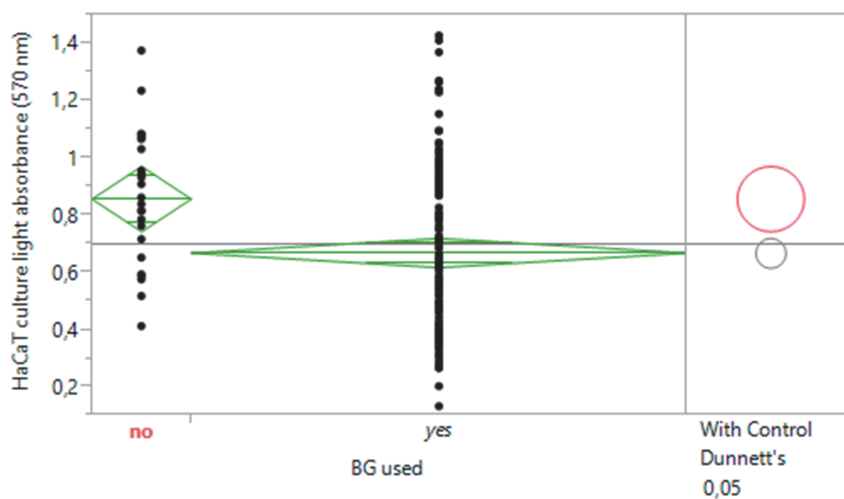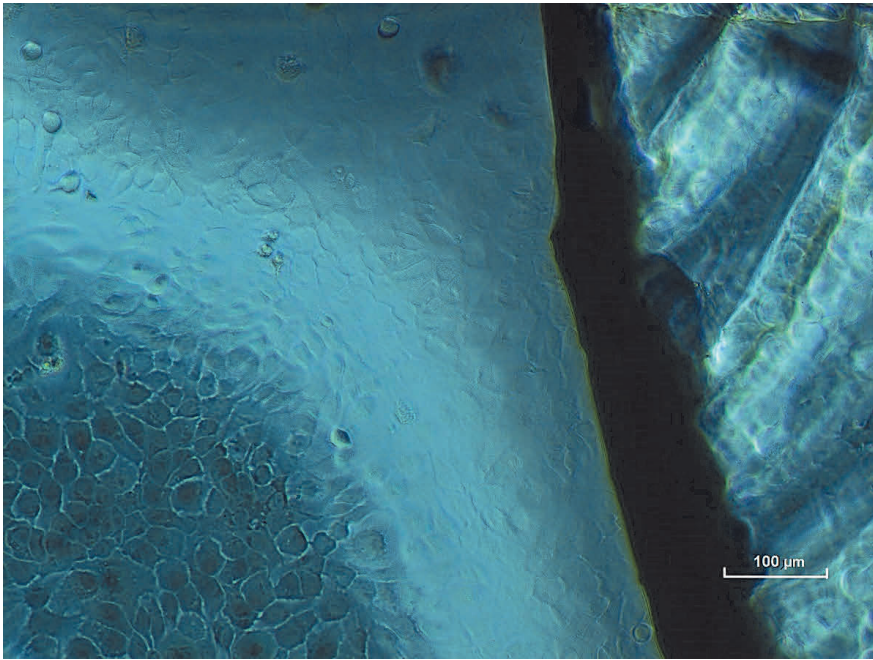

Figure 14. One-way analysis of variance of keratinocyte viability with MTT method. Light absorbance measured with a 570 nm wavelength for cell cultures in KGM without BG and for cell cultures containing 0.5–10 % BG S53P4 in KGM.

Figure 15. BG S53P4 granule (right side of the image) on top of HaCaT keratinocyte layer. Immediate BG contact has promoted keratinocyte cell death. Photo: Jussi Sarin.
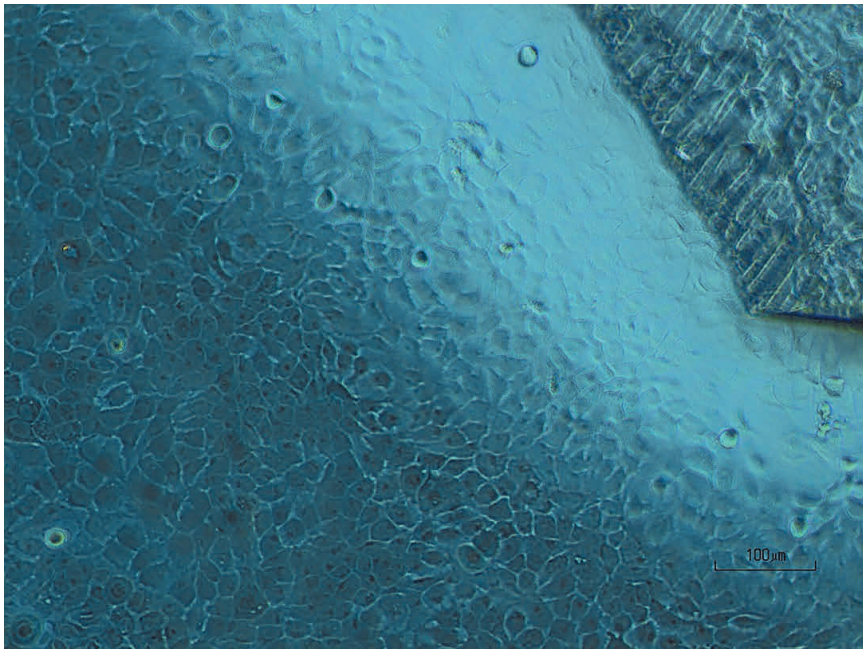
Figure 16. BG S53P4 granule (right upper part of the image) on top of HaCaT keratinocyte layer. Immediate BG contact has promoted keratinocyte cell death. Photo: Jussi Sarin.
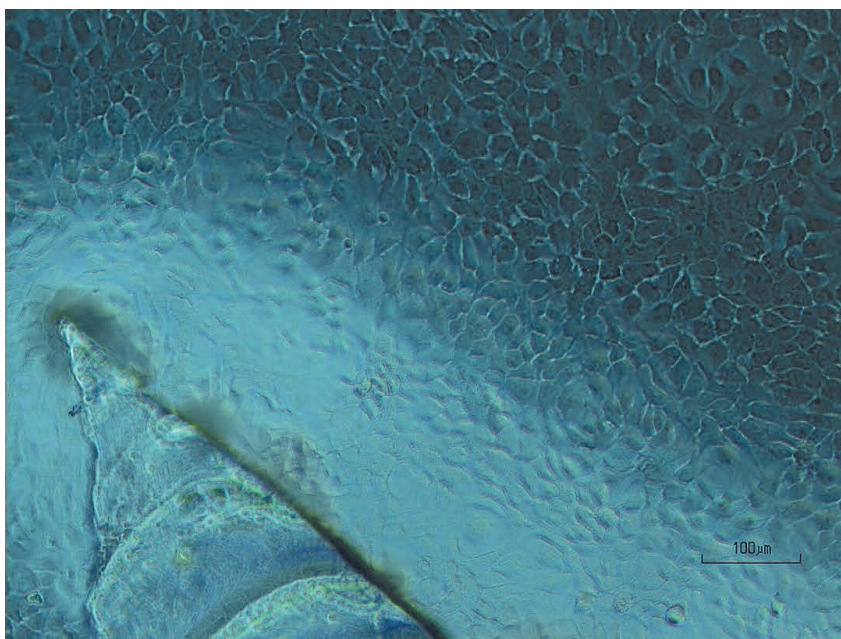
Figure 17. BG S53P4 granule (lower part of the image) on top of HaCaT keratinocyte layer. Immediate BG contact has promoted keratinocyte cell death. Photo: Jussi Sarin.
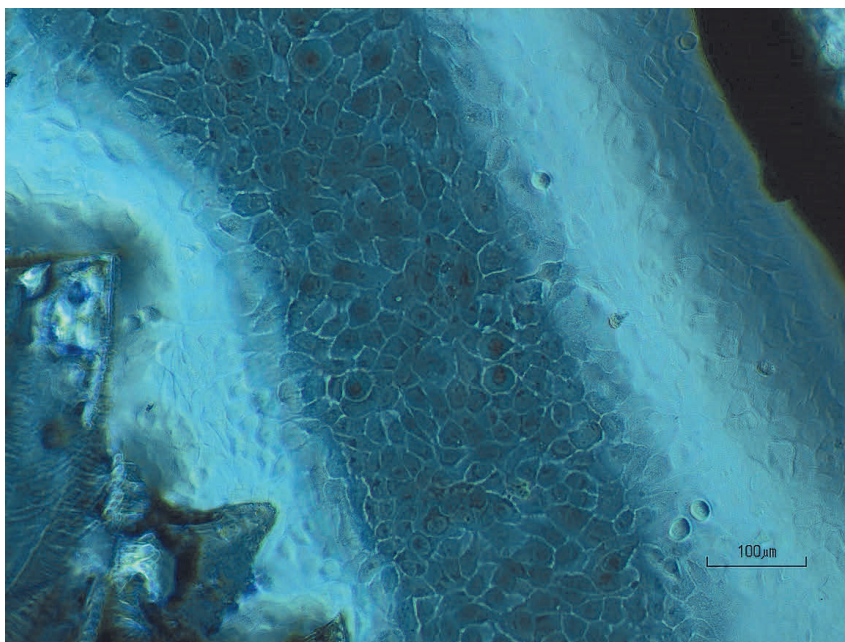
Figure 18. Two BG S53P4 granules (right and left side of the image) on top of HaCaT keratinocyte layer. Viable keratinocytes can be seen between the BG granules. Photo: Jussi Sarin.

6 DISCUSSION

6.1 BG S53P4 as a mastoid cavity obliteration material (I)

In the treatment of cholesteatoma, CWD mastoidectomy often offers an unparalleled surgical exposure of the disease area and the benefit of a wide exposure is related to a low disease recurrence rate (Palva 1973, Osborn et al. 2012). However, several disadvantages, including periodic need for follow-up and cleaning (Kos et al. 2004), favor the endeavor to avoid creation of the radical cavity in the first place; when CWD mastoidectomy is duly needed, studies support either canal wall replacement (Gantz et al. 2005) or mastoid obliteration (Dornhoffer et al. 2008). The possibility of a residual cholesteatoma after the first operation or the otherwise considered need for a second-look operation, however, deters the customary planning of primary mastoid cavity obliteration surgery.

Ideally, mastoid cavity obliteration material should be readily available in sufficient quantities without the need for additional time-consuming graft harvesting surgery. The obliteration material should be bio-compatible, keep its volume for prolonged periods, and be resistant towards microbial pathogens and possible residual cholesteatoma in the mastoid cavity.

Autologous mastoid cavity fillers have long been the golden standard in mastoid obliteration surgery. When revision mastoidectomy is needed, mastoid obliteration with autologous bone paté seems to provide better results with fewer symptoms compared to those of revision mastoidectomy alone (Irving et al. 1994). Recently, Quaranta et al. found that bone paté, collected during mastoidectomy surgery, increased the viability of osteoblasts in vitro (Quaranta et al. 2016). Thus, autologous bone per se is a worthwhile alternative, either as a sole option when adequate amount of patient-derived material is available, or as an addition to a greater volume of synthetic material. However, the problem with autologous bone harvested from the operation area is not only the limited availability, but also the tendency of the graft material to lose some of its volume over time (Abdel-Rahman et al. 2008). Compared to other autologous obliteration materials like free or pedicled muscle grafts, however, bone seems to be the most capable over time (Linthicum 2002).

As far as synthetic mastoid cavity obliteration materials are concerned, BG S53P4 seems favorable when compared to other available synthetic materials discussed in chapter 2.4.2. Antibacterial properties (Stoor et al. 1998, Stoor et al. 1999, Munukka et al. 2008, Leppäranta et al. 2008, Bortolin et al. 2016), bone bonding (Jang et al. 2007, Hench and Jones 2015) and safety profile (Wilson et al. 1981, Bernardeschi et al. 2015) all seem suitable.

In this study, 26 patients were treated with mastoid obliteration, using BG S53P4 granules with fibrin glue fixation. The goal of treatment was to remove cholesteatoma for 16 patients, to eradicate chronic infection without cholesteatoma for 9 patients, and also to close dural fistula with CSF leakage for three patients. The reasonably long average duration of hospitalization, 5.3 days, can be explained by the cautious approach by the surgeons, as bioactive glass obliteration was in an experimental phase during the first patients in the late 1990's. Besides pursuing the goal of creating a dry, safe ear, an important objective was the size reduction of the remaining mastoid cavity. Dry ear or only intermittent otorrhea was achieved with 88 % of patients. Excluding the patients with the shortest follow-up or the patients who were lost to follow-up, 96 % of the patients had a dry ear or only intermittent otorrhea. In addition, the goal of reducing the size of the cavity was achieved with 92 % of the patients. However, this assessment was based on the subjective evaluation of the surgeon postoperatively. This result compares well with other techniques of mastoid obliteration: Ramsey et al. accomplished a dry, trouble-free mastoid cavity in 90 % of their 60 patients using an inferiorly pedicled, periosteal-pericranial flap along with bone paté as an obliteration material (Ramsey et al. 2004). Mokbel and Khafagy published their series of 100 patients with chronic suppurative otitis media treated with CWD mastoidectomy and cavity obliteration using local flaps and bone paté, and reported adequately or perfectly dry ears in all of their patients in 6 year-follow-up (Mokbel and Khafagy 2012). One patient in this study, with 3 previous mastoid operations, had prolonged otorrhea postoperatively, implying a residual infection, still not totally addressed by the most recent surgery. Nevertheless, the operated ear became dry during the following 1.5 years without a need for additional revision surgery in the form of subtotal petrosectomy. It could be hypothesized that this observation is based on the strong antibacterial potential of BG S53P4, as several other challenging bacterial environments have been treated with success using BG S53P4 granules as part of the surgical protocol (Lindfors et al. 2010, McAndrew et al. 2013, Kankare and Lindfors 2016). Indeed, it seems that when microbiological contamination is present, all other synthetic materials used *in vivo* fare poorly, with eventual need for extraction of the implanted material.

When it comes to surgical technique of mastoid obliteration with BG granules, adequate coverage of granules with suitable soft tissues must be emphasized. In the anterior direction towards the ear canal, inadequate fascia and cartilage coverage can cause the exposure of the granules, as was the case with two patients in this study. This topic was also discussed by Silvola, who learned adequate cartilage support as well as Palva-type flap next to outer ear canal skin to be in order, preventing granule exposure into the ear canal (Silvola 2012). Bernardeschi et al. also stressed the importance of sufficient granule coverage in their work, as they found cartilage resorption leading to BG exposure in the ear canal (Bernardeschi et al.

2015). However, unlike Silvola, they considered skin and cartilage to be sufficient barriers for BG granules, without the need for Palva flap.

Cerebrospinal fluid leakage through the temporal bone, a fairly common complication after surgery for acoustic neurinoma, for example, can be treated with total tympanomastoid obliteration (Mehta and Harris 2006). In this study, a total of three patients had dural fistulae with CSF leakage. In addition, with another three patients, a bony dehiscence at the middle cranial fossa was observed with a caudally protruding dura. Since BG granules, as part of occlusion of the fistula and mastoid obliteration, were used with all of these patients, and separate BG plates were used with latter patients as a skull base support towards cranial direction, a reliable tissue-BG S53P4 -compatibility is expected as far as dural contact of BG is concerned. As fascia was used between the dura and the BG in this patient series, there was no immediate BG-dura –contact *per se*. Based on the results by Aitasalo and Peltola with frontal sinus obliteration, BG S53P4 appears to be well tolerated in the proximity of the dura (Aitasalo and Peltola 2007). Due to the limited number of CSF leakage patients in this study, further studies are obviously needed before challenging more traditional means of transtemporal CSF leak management with temporalis fascia and bone grafts, for example (Kari and Mattox 2011).

Although mastoid obliteration with endeavor to restore a correct anatomical shape of the posterior ear canal wall is favorable in regards to hearing *per se* (Jang 2002, Lee et al. 2009, Osborn et al. 2012), the majority of the patients in this study had significant preoperative sensorineural hearing defects. Thus, apart from cochlear implantation, hearing restoration was not possible even in theory for these patients. As a part of the surgery, ossiculoplasty was performed for six patients with notable conductive hearing deficit, with an average of a 4.0 dB hearing gain postoperatively. The postoperative mean PTA of 53.6 dB on the affected ear reflects the toll chronic middle ear infection and cholesteatoma poses on hearing. Hearing results in this series are comparable to those reported by Bizakis et al. with their PTA average of 55 dB in 195 patients treated with CWD mastoidectomy (Bizakis et al. 2006).

6.2 Simultaneous use of fibrin glue and BG S53P4 granules (II)

Since 1976, the use of different types of fibrin glue has traditionally been based on their advantageous hemostatic and clot-forming qualities and relative safety, while the mechanical strength of fibrin glue is typically low (Dickneite et al. 2003, Yoo et al. 2008, Montana et al. 2012). Over the years, owing to the growth factor content of these plasma-derived protein-rich products, a growing interest has emerged to study the potential of fibrin glue in the field of biomaterial composites and tissue engineering. For example, Wang and colleagues used a fibrinogen layer as part of

a polymer scaffold in order to generate smooth muscle cell growth (Wang et al. 2010). Biologically active fibrinogen network has also been a beneficial part of a polymer scaffold, as far as *in vitro* cell growth and adhesion of human umbilical endothelial cells (Gugutkov et al. 2011) and adipose-derived stem cells (Zhao and Wang 2013) are concerned. Recently, Fujioka-Kobayashi and colleagues found fibrin glue to be a suitable carrier for recombinant human bone morphogenetic protein (rhBMP9), a strong osteogenic inducer. In their study, nearly 65 % of rhBMP9 was retained in a fibrin glue scaffold, and the combination of fibrin glue and rhBMP9 favored bone stromal ST2 cell differentiation towards osteoblasts (Fujioka-Kobayashi et al. 2016).

Although fibrin glue has been in clinical use over four decades, its use with several bone substitutes, including allograft bone, coral granules and hydroxyapatite, has been controversial as far as positive and negative influences are concerned (Le Guéhennec et al. 2004). BG, impregnated with fibrin glue and implanted into a muscle pouch, resulted in neo-osteogenesis in a mouse model by Abiraman and colleagues, while uncoated BG did not show any osteoinduction (Abiraman et al. 2002). In another study, fibrin glue mixed with bone pate, increased osteoblast cell viability *in vitro* (Quaranta et al. 2016). In a recent work done by Lappalainen et al., fibrin glue was found to be a suitable addition to a filler material in rabbit calvarial bone defects, as far as new bone formation, studied by micro-CT imaging, was concerned. However, BG scaffolds and β-TCP granules fared worse than autologous bone grafts (Lappalainen et al. 2016).

In the first part of Study II, the fibrin glue penetrated to a depth of a few millimeters in the bed of the BG granules, when granules between 0.5 and 0.8 mm in size, suitable in mastoid obliteration surgery, were used. In clinical use, after BG granules have been moistened with a physiological saline solution before application as bone cavity filler, additional use of fibrin glue results in a firm mixture that some surgeons find easier to control (Stoor et al. 2010, Silvola 2012). Considering that the required total volume of the BG granules was up to 20 cm^3 per patient in study I, there is a chance that the applied fibrin glue remains on the uppermost layer of BG granules, while the more distal granules might remain unaffected. In order to obtain the maximum stability of the BG granules, it seems justifiable to either add the glue with the tip of the application cannula deep inside the BG granule layer, or apply BG granules and fibrin glue in a layer-by-layer manner, to ensure as thorough glue permeability as possible.

Antibacterial properties of BGs (Stoor et al. 1998, Stoor et al. 1999, Munukka et al. 2008, Leppäranta et al. 2008, Lindfors et al. 2010 (A), McAndrew et al. 2013, Coraça-Huber et al. 2014) – especially notable with BG S53P4 (Zhang et al. 2010) – are most important in their intended use as a reliable bone cavity filler. The rise in pH appears to be an essential contributing factor as far as antibacterial effects

of BGs are considered (Zhang et al. 2010). Any intervention that reduces the responsive BG surface area and thus reduces the elevation of pH and the osmotic pressure by way of reducing sodium, calcium, phosphate and silicate ion release from the BG surface – which leads to suppression of bacterial growth – has to be considered carefully. The magnitude of bacterial growth suppression depends on the size of the BG granules: the smaller the particles in contact with the surrounding liquid environment, the larger the reactive surface area per weight unit of BG, the faster the ion release rate and the higher the antibacterial effect (Zhang et al. 2008, Massera and Hupa 2014).

In this study, coagulated fibrin glue particles alone did not change the pH of SBF. In contrast, the pH of SBF increased by 0.4 units during a 7-day incubation period, when BG S53P4 granules were used at a concentration of 4 mg/ml. This pH change is in line with other studies with various BG concentrations (Varila et al. 2012, Massera and Hupa 2014). When BG granules, coagulated with fibrin glue, were incubated for 7 days, a pH increase of 0.5 units was observed. When compared to the 0.4 unit pH change of the BG-alone group at several time points, a statistically significant difference was noted (p=0.008, rm ANOVA). In a 14-day test protocol with BG-fibrin glue-combos as part of an ion dissolution test, a 0.6 unit pH increase in SBF was observed after a two week incubation period with a BG concentration of 25 mg/ml. According to these results, it seems that the use of fibrin glue as a coating for BG granules will not diminish the pH increase of SBF. On the contrary, the pH increment was greater in the BG-fibrin glue –group compared to the BG-group. Although further research is needed in order to understand the ability of fibrin glue to bind hydrogen ions and contribute to the pH change of the surrounding liquid environment in an alkaline direction, the antibacterial effects of BGs seem to still be functioning when fibrin glue is used together with BG granules.

In the 14-day test protocol with the BG-fibrin glue –samples in this study, a rapid Ca-ion concentration increment was observed as the early Ca-ion dissolution from the BG surface was at its peak. Later, Ca-ion concentration declined, representing the phase where the CaP layer is growing on the BG surface (Varila et al. 2012). When ion concentration behavior of these samples is compared to results of Massera and Hupa (Massera and Hupa 2014), a slightly slower Ca-ion increase with a prolonged concentration increment phase was observed in this study. To be specific, with a BG concentration of 1.5 mg/ml Massera and Hupa observed a maximum Ca-ion concentration value after 30 hours of SBF incubation, after which Ca-ion concentration declined to a relatively steady state after 70 hours. In this study with a BG concentration of 25 mg/ml, maximum Ca-ion concentration was achieved after two days, and a steady state after Ca-ion concentration decline was observed as late as after 9 days. The difference between these two studies could be

explained by the diffusion barrier between the glass and the solution, produced by the fibrin glue, rendering some of the BG granules less prone to release ions in the solution during incubation. However, as most of the fibrin glue has dissolved already after two days of incubation documented by weight measurements of the BG-fibrin glue –samples, the CaP layer formation process is able to consume Ca-ions from SBF, albeit in a slower fashion compared to a situation without fibrin glue.

The highest silicon concentration was observed after 9 days of incubation in the BG-fibrin glue –protocol, ion concentration increase taking considerably more time compared to earlier work using only BG S53P4 particles without fibrin glue (Massera and Hupa 2014). As far as other ions in SBF are concerned, K, Mg and Na stayed on a proportionately steady level with a very slight decline, whereas P-ion levels declined prominently. The concentration changes suggest that in addition to Ca and P, also K, Mg and Na ions are incorporated into the CaP layer, and ion concentration behavior in this study is comparable with data from previous studies (Nganga et al. 2012, Massera and Hupa 2014). Overall, as fibrin glue fills, at least to some extent, free spaces between the BG granules, slower dissolution reactions of BG are logical. *In vivo*, however, the continuous flow of fluids, as opposed to the static SBF conditions in this study, is likely to enhance the dissolution reactions of BG.

When it comes to the reliability of the results of study II, possible error sources include pH variation between different SBF batches. Therefore, a greater emphasis should be placed on the pH increments, instead of merely observing the final pH values at different incubation protocols. In comparison, the weighing of BG particles was very accurate, whereas weighing of the applied fibrin glue was more challenging. In addition, during SBF incubation, weight measurements of the partly dissolved BG-fibrin glue –combinations are not exact *per se*, as BG particles became, via fibrin glue dissolution, increasingly detached.

6.3   N-butyl-2 cyanoacrylate tissue adhesive as a fixation method of BG products (III)

Cyanoacrylate glues, after first appearing on the market as a means of skin wound closure, have since gained an array of other clinical off-label applications (Spotnitz and Burks 2012). Based on the recent literature, CA glue presents itself, for example, as a valid method of mesh fixation in hernia repair (Matikainen et al. 2016), a method to control bleeding of gastric varices (Weilert and Binmoeller 2016) and a possible method to induce preoperative embolization of a meningeoma with a very dilute glue mixture (Ohnishi et al. 2016). In addition, as a case report level, CA glue has been a fixation method of choice in an isolated cricoid cartilage fracture

(Standlee and Rogers 2016) and a possible alternative for the sealing of a coronary artery perforation (Mishra et al. 2016). Increased use of CA adhesives in surgical disciplines has also brought attention to their disadvantages: intracranial glue embolization of arteriovenous malformation carries a risk of glue misplacement (Fahed et al. 2016), and colonic end-to-end anastomosis in rats were weaker after CA glue application over sutures, compared to standard sutures alone (Güngör et al. 2016). Such an unfavorable effect of CA for the healing process of soft tissues was also observed by Toriumi and colleagues (Toriumi et al. 1991).

When a specific synthetic glue type was chosen for study III, the least amount of tissue toxicity for the chosen product was emphasized. Hence, the position of n-butyl-2-cyanoacrylate as a bio-compatible bone and cartilage adhesive appeared strong (Toriumi et al 1990, Toriumi et al. 1991). Recently, results by Kang and colleagues with *in vitro* cell cultures and *in vivo* have further highlighted the lack of toxicity of n-butyl-2-CA (Kang et al. 2016). As far as the special circumstances during mastoid obliteration surgery are concerned, close proximity of the cavity filler to dura is obvious; therefore, observations made by Shermak and colleagues are of utmost importance, as they found butyl-2-CA glue to cause no significant inflammatory or necrotizing responses, even in direct contact with the surface of rabbit brain (Shermak et al. 1998). Recently, Sohn et al. compared a short-chain cyanoacrylate (SCCA), namely ethyl-2-cyanoacrylate with a long-chain cyanoacrylate (LCCA) of butyl-cyanoacrylate, and discovered LCCA-treated mice having marked bone regeneration at exposed cranium. However, with SCCA-treated mice, such bone regeneration was absent and cranial bone density was significantly lower than in control or LCCA-treatment groups (Sohn et al. 2016).

In this study, cylindrically shaped BG S53P4 particle - butyl-2-CA glue –composites were subjected to compression strength measurements. When the compression begins, surface stress increases in a linear manner (Figures 9 and 10). Following this elastic phase, during which the longitudinal movement of the compression device is quite small, a yielding point of 15-25 MPa is ultimately achieved, where the specimen tends to sag in a non-elastic manner instead of breaking. No statistically significant difference in compression strength between different specimen groups with various water immersion periods were noted, the mean stress at maximum load ranging from 28.6 to 36.3 MPa. However, water immersion of BG-glue –composites led to increased Young's modulus values. For the 3- and 10-day immersion groups, compression modulus of the specimens, compared to the dry specimens, was greater in a statistically significant manner. For the 30-day immersion specimen group, the mean modulus was still higher than that of the dry specimen group, but the difference was not statistically significant (p=0.36).

Based on the results of this study, butyl-2-CA glue seems to keep the glue-BG – composite solid for at least 30 days in spite of water exposure. Interestingly,

Young's modulus of the butyl-2-CA glue composites even increases after water immersion. An explanation for this behavior could be that after the LCCA glue is exposed to any tissue or other substance containing water, leading to a very rapid exothermic reaction where glue monomers are linked into long polymers, some slower reactivity of cross-linking might still take place strengthening the composite further. Just very recently, Li and colleagues studied the early and delayed polymerization process of cyanoacrylate glues with different viscosities, and were able to introduce a new method to document the process in a reproducible manner (Li et al. 2017). Although modulus of the glue-BG –composites finally decreased after 30 days of water immersion, compared to the 3- and 10-day-values, CA glue seems able to produce a solid, rather impermeable barrier on the composite surface without any notable water absorption into the composite core, retaining mechanical strength of the composite. Contrary to the results of Oral and colleagues (Oral et al. 2014), who observed several types of BG particulate filler composites gaining weight throughout the 60-day water storage period, the water sorption percentage of the composites in this study reached its maximum value after only three days' water immersion.

The CA glue's potential to produce strong adhesion has been studied earlier in medical disciplines from other perspectives. CA glue produced a nearly four-fold tensile strength compared to a biological adhesive, when overlapping samples from calf pericardium were glued together (García Páez et al. 2004). Kull et al. also found the adhesive properties of n-butyl-2-CA superior to fibrin glue for all adhesion tests with pig skin samples (Kull et al. 2009). As far as bone fracture attachment is concerned, butyl-2-cyanoacrylate glue by itself, or in concert with plates, seems to work well with compressive forces as reported by Gosain and colleagues; on the other hand, with distractive forces, traditional screws and plates were stronger than glue fixation (Gosain et al. 1998). Ahn et al. also found, studying the load-bearing capability of biodegradable plates on frontal bone osteotomy sites in pigs, that the use of butyl-2-CA adhesive was comparable strength-wise to metal plates and screws (Ahn et al. 1997).

BG by itself, in its granule form, is not an optimal material for load-bearing bone defects (Hulsen et al. 2016). N-butyl-CA glue appears to be, however, a synthetic tissue adhesive that used in conjunction with BG granules offers a strong mechanical adhesion, compared to biological adhesives. As it lacks tissue toxicity and seems to retain its adhesion strength in an aqueous environment as part of a BG-containing composite, at least in the short term, it might present a possible alternative for solid BG composite implant fixation where implant-bone –interface strength requirements are considerable. However, in mastoid obliteration surgery with the use of BG particles, biological tissue adhesive seems to offer adequate mechanical properties for successful surgery (Study I). Hence, other qualities of adhesives that are considered to be used with BG granules play a decisive role.

In Study III, maximum SBF pH of 7.9 was observed when only BG granules were incubated. Similarly to results of Study II, fibrin glue and BG granules, used in conjunction, produced a greater SBF pH change at the end of the test protocol, when compared to BG granules alone. The use of CA glue, however, had a considerable reducing effect on the SBF pH increase. Indeed, final pH in the CA glue-BG particle –group was only 7.5. It seems that CA glue, as it polymerizes rapidly on the glass surface, forms a solid diffusion barrier reducing notably BG granules' reactive surface. Fibrin glue, on the other hand, is rapidly degradable as shown in Study II, and allows for a quick glass surface – SBF solution ion exchange and subsequent pH increase.

Since the antibacterial effects of BG are dependent on the pH increase of the surrounding liquid environment (Munukka et al. 2008, Zhang et al. 2010), it would be favorable if the method of BG fixation had as little interference with the BG's ability to dissolve as possible. As far as relatively large bioactive fiber-reinforced composite implants are considered, as used by Aitasalo et al. in order to compensate for cranial bone loss after neurosurgery (Aitasalo et al. 2014), one might question, if CA glue fixation was to be considered, would the reduced solubility of BG at the CA glue seam area be a clinically relevant phenomenon. Standard screw fixation with supposedly inert titanium material should, in comparison, have no effect on the local BG solubility. CA glues do have, however, antibacterial effects towards gram positive bacteria (Quinn et al. 1995, Wilkinson et al. 2008, Romero et al. 2009). As BG granules typically do not require ultimate fixation strength in bone cavity filling, observed solubility behavior of CA glue –BG S53P4 –composites in this study leads to the conclusion that cyanoacrylate glue is not likely an optimal adhesive with BG granules in mastoid obliteration surgery.

An alkaline liquid environment, produced by BG surface reactions, is clearly antimicrobiologically advantageous. However, if the pH reaches excessively high levels, new bone formation is jeopardized. Monfoulet and colleagues reported excessive alkalization of the microenvironment resulting in poor osteogenic response both *in vitro* and *in vivo*, using several tissue engineered constructs containing coral- and hydroxyapatite / tricalcium phosphate as well as BG 45S5 (Monfoulet et al. 2014). More specifically, they observed the cessation of human bone marrow-derived mesenchymal stem cell (hBMSC) proliferation when pH 8.85 was exceeded, and the cells eventually died at pH 9.37. In addition, they found that the pH in the core environment of hBMSC-containing constructs stayed lower in the region of 8, whereas cell-free BG 45S5-constructs showed a more alkaline inclination with pH values exceeding 9. As far as well-being and function of osteoprogenitor cells are concerned, the highest pH values in this study (II-III), with chosen BG particle size and concentration values in SBF, were notably lower than those

needed to induce hindrance to hBMSCs for both the BG particles only –group and fibrin glue-BG particle group, not to mention the CA glue-BG particle –group.

6.4   The effect of BG S53P4 on immortalized HaCaT keratinocytes (IV)

Immortalized human HaCaT keratinocytes have not only proven their worth as a viable cell model in skin toxicology studies and as a model of wound healing (Henseleit et al. 1996, Kleszczyński et al. 2013, Guo et al. 2015, Arteaga-Gómez et al. 2016, Ferreira et al. 2016, Sun et al. 2016, Kim 2016), but they also have been used as an *in vitro* model of cholesteatoma (Laeeq and Faust 2007). Albeit HaCaT research has been quite extensive in the field of dermatology, so far there has been no published data of HaCaT cell viability in an alkaline environment or even more specifically, what kind of a reaction, if any, does BG yield from keratinocytes. There is, however, information available regarding the behavior of another relevant cell type in human primary cholesteatoma, namely fibroblasts, in the immediate vicinity of BG 353P4 granules. In their work, Detsch and colleagues cultured CCD-18CO fibroblast cells with BG S53P4 granule contact, in order to measure the release of vascular endothelial growth factor from the fibroblasts (Detsch et al. 2014). With a BG S53P4 granule concentration ranging from 0 to 1 percent, they did not notice any alterations in fibroblast cell morphology or a reduction in cell viability. From the perspective of osteointegration and osteoinduction between BGs and bone, and also in regards to antibacterial effects of BGs, the pH and ion changes in simulated body fluid, that BG products are able to produce as they slowly dissolve, have been widely studied (Leppäranta et al. 2008, Munukka et al. 2008, Zhang et al. 2009, Zhang et al. 2010). However, such data has not been available regarding culture media of mammalian cells.

The hypothesis that Study IV was based on, i.e. BG S53P4 has a growth inhibiting effect on keratinocytes, rose from the clinical observations with BG S53P4 granules and mastoid obliteration surgery in general. Recurrence of the cholesteatoma is possible (Luers and Hüttenbrink 2016), and when a decision of mastoid obliteration is made, following cholesteatoma removal, a substantial clinical advantage could be gained if the obliteration material had properties that could prevent the re-occurrence of the disease. When the postoperative well-being of the normal skin and to be specific skin keratinocytes, on the other hand, are concerned, the obliteration material should not have an outright toxic effect on keratinocytes or any normal cells in general.

In this study, the pH of cell culture media DMEM and KGM rose as BG S53P4 granules were incubated in the media. Depending on the BG concentrations with

a range of 0.5–10 %, pH increments of up to 1.00 and 1.23 were observed, respectively, yielding the final pH values of 8.43 and 8.58 after an incubation period of six days. When these two media are compared, it seems that KGM has a more prominent buffering capacity in the concentration area of 0.5–2.5 % BG, whereas the pH curve in the DMEM solution group was more straightforward (Figure 12). In the MTT cell viability assay, the BG-DMEM and BG-KGM dilutions without the actual BG granules at the time of cell culture usage, appeared to reduce the viability of HaCaT keratinocytes as a whole. In the DMEM group, the lowest calculated HaCaT viability of 73 % was observed in the 10 % BG group, whereas the lowest viability of 40 % was observed in the 2.5 % BG-KGM group, respectively. When all BG groups were combined, HaCaT viability was higher in a statistically significant manner both in DMEM and KGM controls compared to BG-containing solutions. A clear dose-response-relationship was not seen in the MTT test in the BG-KGM group, as the linear regression analysis did not deliver a particular linear correlation. However, in the BG-DMEM-group there was a concentration-dependent linear viability response in the linear regression analysis, as presented in chapter 5.4.

Based on the light microscopy findings of the HaCaT cultures, direct BG S53P4 granule contact not only appears to promote keratinocyte cell death, but BG also seems to discourage keratinocyte proliferation in the scratch assay test. In static cell culture solutions, local pH should be the highest in the proximity of the BG surface. The results of the MTT cell viability test with different pH values of the culture media and the findings of the granule-HaCaT-contact tests suggest that the mechanism that affects HaCaT viability and proliferative ability might depend on pH. An excessively alkaline environment is not productive for cell growth or cell culture maintenance, as demonstrated by Monfoulet et al. with human bone marrow-derived mesenchymal stem cells. They noticed the cessation of cell proliferation of hBMSCs with pH value of 8.85 and eventually witnessed cell death at pH 9.37 (Monfoulet et al. 2014). These findings very much place the uppermost pH boundaries for BG-containing composite products, if their unique capability to promote osteogenesis is still to be retained. In addition to the usual extracellular influence mechanism of BG products, human bone marrow and adipose-derived stem cells are capable of uptaking very small BG particles, as demonstrated by Tsigkou and colleagues. In such a case, intracellular dissolution of BG particles with a diameter of approximately 200 nm does not hinder metabolic activity with an intracellular BG concentration of 0.005 %, but greater concentrations of 0.01 and 0.02 % produce a small decrease in cellular metabolism (Tsigkou et al. 2014).

HaCaT cell death in direct BG S53P4 granule contact is more likely to be apoptotic than necrotic in nature. To support this theory, the mean concentration of both interleukines 6 and -8, analyzed from the medium samples that were taken from

the same culture wells that first underwent BG granule exposure and subsequent microscopy, were lower in the 2.5 % BG group than in the 0 % BG group (p=0.0038 and p=0.0013, respectively). When cellular damage leads to cell necrosis, cytoplasm is being released from the dying cell and the concentration of pro-inflammatory cytokines in the surrounding liquid environment could be expected to escalate. Hence, it could be speculated that the IL-6 and IL-8 data of Study IV are caused rather by the process of cellular apoptosis: in apoptosis, cytoplasm is contained within a dying cell (Allen et al. 1997). Therefore, a greater number of active keratinocytes in the 0 % BG-group might be able to release more IL-6 and -8, as opposed to the reduced cell population in the BG-containing cultures. Nonetheless, there was no statistically significant difference in the MTT viability experiment between 0 % BG and 2.5 % BG groups. This could be related to the fact that there were no BG particles themselves in the MTT assay wells, only BG solubility products with a pH difference of 0.53 between the groups. At any rate, more data is needed before further conclusions can be made of HaCaT cytokine release and the cascade of HaCaT cell death, promoted by the vicinity of BG S53P4 granules.

How does the published clinical data compare with the *in vitro* results of Study IV? Considering the width of the dead keratinocyte layer in contact with the BG granules, 8–10 cells or approximately up to 0.4 mm in thickness, one might expect some form of skin irritation to take place *in vivo* resulting from the BG contact. Indeed, the usual dimensions of the normal human skin epidermis are of the same magnitude compared to this affected keratinocyte layer, depending on the anatomical position of the skin (Laurent et al. 2007). After mastoid obliteration surgery with BG S53P4 granules as the obliteration material of choice, both Silvola and Bernardeschi and colleagues noticed on their part that there is marked skin irritation in the operation area 1-4 weeks postoperatively (Silvola 2012, Bernardeschi et al 2015). However, with later follow-up visits, the condition of the skin in the ear canal and the retroauricular area returned to normal. As their conclusion regarding the operative technique, both authors consider adequate cartilage usage between the BG granules and the skin important.

Could the Ca^{2+} levels play a decisive role in the reduction of keratinocyte viability? As BGs are incubated in SBF, for instance, the concentration of Ca, Na, K, Mg, Si and P ions increases in general. The rate of the concentration increment of ions depends largely on the total reactive surface area of the incubated BG products, and obviously a powdered form of BG is able to provide the greatest reactive surface area per weight unit. In addition, as far as BG scaffold structures are concerned with a porous surface, increments in pore diameter favor more rapid ion release (Jones et al. 2006). Zhang and colleagues observed ion concentration changes in SBF using six different types of BGs in powder form, using 10 % BG solutions. Interestingly, S53P4 appeared quite unique in its solubility behavior

compared to other types of glasses: initially there was a Ca^{2+} increment of the solution in the first few hours of incubation, which was then followed, differing from other BGs, by a decline in Ca^{2+} levels. The total range of Ca concentration values was 3-fold, from 1.3 to 3.9 mmol/L. SBF pH was also highest in the S53P4 group, both in the powder bed and in the mixed solution (Zhang et al. 2010). Although the buffering capabilities of cell culture media used in this study, DMEM and KGM, presumably differ from that of SBF, it seems safe to assume that Ca concentration changes in culture media do not reach the same 3-fold magnitude described above. This assumption is based on the observation that in study IV the highest pH value was 8.58, when BG granule concentration was 10 %. In comparison, in a work done by Zhang et al., the highest pH value exceeded 11.5. The question whether Ca levels of the culture media are able to affect keratinocyte cell viability, has been reflected previously. A culture medium with only 1/13th of the normal medium Ca concentration caused an over 10-fold reduction in cholesteatoma migration rate, when primary cholesteatoma keratinocytes were cultured. Nevertheless, this reduction in medium Ca level did not promote cell death *per se*, as the keratinocytes matched the growth rate of the control sample after replacing the medium with normal Ca content (Minotti el al. 1996). Equally, calcium channel blocker verapamil reduced the cholesteatoma migration rate to near zero at a concentration of 300 µg/L in α-MEM cell culture medium, while eventually promoting keratinocyte death with a concentration range of 400–600 µg/L (Kountakis et al. 2000).

There are a few possible sources of error in Study IV. The scratch assay test is susceptible to interpretation bias and is not able to produce, even at its best, exact quantitative data. Roshan et al. recently discussed the two modes of human keratinocytes, that are interconvertible based on the confluence status of the cell culture. Confluent, or the balanced phase of keratinocytes is replaced by the expanding phase of cells, in response to suitable stimulus, such as a scratch wound (Roshan et al. 2016). In order to minimize the possibility of result variation based on this cell mode alternation, a considerably larger amount of culture plates might be needed. To minimize the possibility of error in the MTT cell viability test, a large number of samples were obtained for each BG-concentration group. In addition, absorbance values of the HaCaT cultures were compared against blank DMEM and KGM controls in addition to the base absorbance values of empty wells, before viability percentages were calculated. Finally, strong clinical conclusions on the basis of these results should be made with caution. There are several factors causing uncertainty in a straightforward *in vivo/in vitro* –comparison: a monoclonal, monolayer keratinocyte cell model differs substantially from the clinical reality, while the nature of the liquid environment, i.e. static versus dynamic as well as volume of the liquids have to be considered as confounding elements when extrapolating these results.

7   CONCLUSIONS

On the basis of the retrospective clinical study, *in vitro* –material studies with tissue adhesives and BG and the keratinocyte cell study, the following conclusions are drawn:

1.   BG S53P4 granules can be used alone or together with bone in mastoid cavity obliteration surgery when such an operation is needed, either after previous canal wall down surgery or as a primary treatment modality.

2.   Fibrin glue does not interfere with the solubility of BG S53P4 granules in a way that would hinder the important antibacterial properties of BG.

3.   N-butyl-2 cyanoacrylate tissue adhesive provides a strong adhesion for BG and is able to withstand water exposure at least 30 days without losing its rigidity. However, as it forms a diffusion barrier on top of the glass surface, solubility of BG is greatly reduced. Hence, cyanoacrylate glue does not seem a justifiable option as a tissue adhesive in mastoid obliteration surgery. Further studies are needed in order to realize its suitability as a possible tool for rigid BG implant fixation.

4.   BG S53P4 appears to inhibit HaCaT keratinocyte growth and reduce HaCaT viability. In addition, the immediate neighborhood of BG granules seems to promote HaCaT apoptosis. The observed effect is limited and may be advantageous for cholesteatoma treatment.

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