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Dissertation

zum Erwerb des Doctor of Philosophy (Ph.D.) an der

Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

Disrupted biomineralization in zebra mussels after exposure to bisphenol-A: Potential implications for molar-incisor hypomineralization and Evaluation of isobornyl acrylate content in medical devices for diabetes treatment

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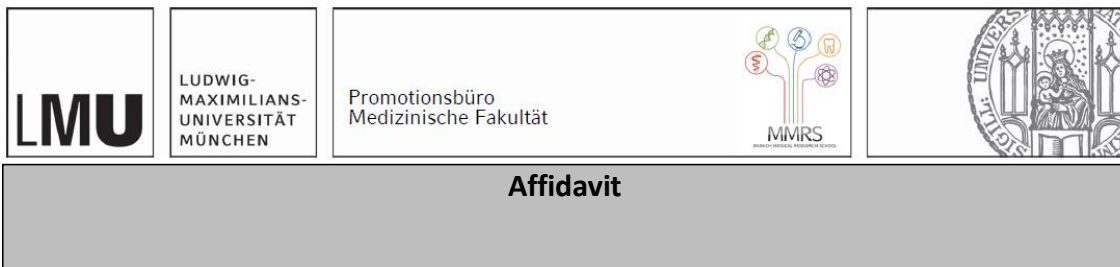**Third evaluator:** Prof. Dr. Peter Thomas

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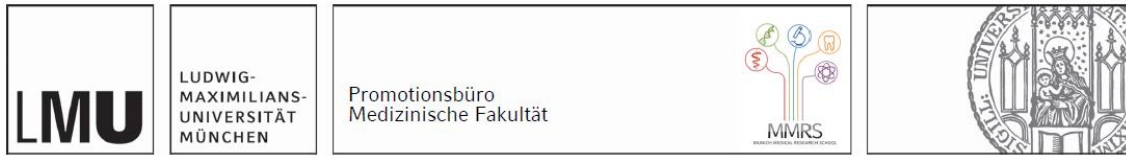
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Table of content

List of abbreviations

Paper I

MIH	Molar incisor hypermineralization
BPA	Bisphenol-A
LC50	Median lethal concentration at 96 h
CI	Confidence interval
MMA	Methylmethacrylate

Paper II

CGM	Continuous glucose monitoring
CSII	Continuous subcutaneous insulin infusion
ACD	Allergic contact dermatitis
IBOA	Isobornyl acrylate
GC	Gas chromatography
CF	Caffeine
GC/MS	Gas chromatography-mass spectrometry
LOQ	Limit of quantification

List of publications

1. Publication for Cumulative Dissertation

Liu FF, Reichl FX, Milz S, Wölfle UC, Kühnisch J, Schmitz C, Geist J, Hickel R, Högg C, Sternecker K. Disrupted biomineralization in zebra mussels after exposure to bisphenol-A: Potential implications for molar-incisor hypomineralization. Dent Mater. 2022; 38(4), 689-699.

Kamann S, Oppel E, **Liu FF**, Reichl FX, Heinemann L, Högg C. Evaluation of Isobornyl Acrylate Content in Medical Devices for Diabetes Treatment. Diabetes Technol Ther. 2019; 21(10), 533-537.

2. Further Publication

Liu FF, Reichl FX, Milz S, Wölfle UC, Kühnisch J, Schmitz C, Geist J, Hickel R, Högg C, Sternecker K. Vitamin D3 application reduces bisphenol A-induced hypomineralization in shells of zebra mussels. (Under preparation)

Oral presentation: **Liu FF**, Reichl FX, Milz S, Wölfle UC, Kühnisch J, Schmitz C, Geist J, Hickel R, Högg C, Sternecker K. Bisphenol-a disturbs bio-Mineralization in mussels: Potential implications for molar-incisor hypomineralization. In *J Dent Res Vol* #(Spec Iss_): 0054, (https://iadr.abstractarchives.com/abstract/ced-iadr2021-3597539/bisphenol-a-disturbs-bio-mineralization-in-mussels-potential-implications-for-molar-incisor-hypomineralization)

Reichl FX, Högg C, **Liu FF**, Schwarz M, Teupser D, Hickel R, Bloch W, Schweikl H, Thomas P, Summer B. Actovegin® reduces PMA-induced inflammation on human cells. Eur J Appl Physiol. 2020; 120(7), 1671-1680.

1. Your contribution to the publications

1.1 Contribution to paper I

I am the independent first author in the first study. I was involved study design. I performed shells staining with calcein in zebra mussels and exposed mussels with bisphenol-A (BPA), cadmium, doxycycline, erythromycin and doxycycline, then collected shells. I conducted all histological process for shells including dehydration, degreasing, embedding, as well as shells cutting and slides preparation. Took photographs for all shells using the confocal microscope and measured fluorescence intensity of calcein and doxycycline in shells. Additionally, I carried out the statistical analysis and produced all the figures and tables included in my paper. I wrote the first draft of the manuscript and worked with all co-authors to revise and finalize it.

1.2 Contribution to paper II

I worked as a co-author in the second paper included in this thesis. I prepared the samples and eluted them with water and methanol for 3 days. I performed the measurement of Isobornyl acrylate (IBOA) content in the water and methanol eluates of the different samples. In addition, I was involved in the discussion of results and revision of manuscript.

2. Introductory summary

2.1 Summary of the PhD project

I performed my PhD projects from October 2018 to October 2022 at the Department of Conservative Dentistry and Periodontology, Walther-Straub-Institute of Pharmacology and Toxicology and Institute of Anatomy at Ludwig-Maximillian-Universität München, supervised by Prof. Dr. Dr. Franz-Xaver Reichl. Two studies were conducted and published to accomplish my Ph.D. program.

Molar incisor hypermineralization (MIH) is a systematic developmental defect of enamel in one or more first permanent molars and is present with or without incisor involvement, and can be caused by a disruption in the calcification [1]. The severe MIH present with crown destruction, enamel breakdown, history of tooth sensitivity and aesthetic problems, which can adversely affect quality of patient life and challenging dentists to treat [2]. Nevertheless, the estimated global prevalence of MIH is 13.5%, of which moderate to severe MIH prevalence is as high as 36.3%, although the prevalence varies considerably by country [3]. MIH clearly deserves more attention as a global dental public health issue. However, the aetiology of MIH remains unclear, because the evidence of existing study is low, as well as common vertebrate models are expensive and subject to ethical restrictions [4, 5]. Hence, it will be a great progress on etiology research of MIH to find a new, simple, inexpensive animal model that is not subject to ethical restrictions. In the last decades, invertebrate models have been evaluated and considered as valid models in biomedical research, principally because of their biomineralization of the carbonate shell during growth and ethical restrictions on vertebrate utilization [6, 7]. Of these, zebra mussels (*Dreissena polymorpha*) have a high reproductive rate as it can occur at great density [8]. Furthermore, zebra mussels were established as a biomineralization model for bone growth [7]. Thus, the first project aimed to explore the feasibility of zebra mussel as a new biomineralization model for screen potential MIH-causative factors by investigating the effect of teeth enamel mineralization interferers on biomineralization in mussels. This work was published as follows: Fangfang Liu, Franz-Xaver Reichl, Stefan Milz, Uta Christine Wölfle, Jan Kühnisch, Christoph Schmitz,

Jürgen Geist, Christof Högg, Katharina Sternecker. Disrupted biomineralization in zebra mussels after exposure to bisphenol-A: Potential implications for molar-incisor hypomineralization. *Dental Materials*, Dental Materials. 2022; 38(4), 689-699.

Skin reaction has been reported in diabetic patients because of the external usage of continuous pumps for continuous subcutaneous insulin infusion (CSII) and glucose monitoring (CGM) systems. Study found 63% of CSII and 46% of CGM users (2-20-year-old patients) were currently experiencing visible skin reactions at one or more sites, in which eczema and pruritus were the most frequent skin symptoms; patients commented worse on skin problems associated with CGM than those associated with CSII [9]. Skin irritation and hypersensitivity are caused by CGM adhesives [10-12]. Although skin irritation is a common symptom, it does not always occur with every usage. Furthermore, skin irritation is usually due to factors of individuals (e.g., predisposition to dry skin or age) and physical (e.g., skin occlusion and sweating under sensor, and epidermal damage when removing the adhesive) [13]. Allergic contact dermatitis (ACD) is not as common, nevertheless is of greater clinical significance. Isobutylene acrylate (IBOA) has been reported that is an allergen causing ACD in diabetic patients using the Freestyle Libre system, a newly introduced glucose sensor [14, 15]. Therefore, IBOA may be released from the plastic material of Freestyle Libre itself and then spread out through the adhesive to the skin. Currently, the incidence of ACD due to IBOA is not well-estimated, which is partly because ACD typically occurs months later after use of Freestyle Libre. Furthermore, skin reactions (in particular) related with use of medical devices for diabetes treatment have not been well described. Thus, the second study aimed to evaluate the IBOA contents of some medical products that are applied to skin for diabetes treatment. This work was published as follows: Stefanie Kamann, Eva Oppel, Fangfang Liu, Franz-Xaver Reichl, Lutz Heinemann, and Christof Högg. Evaluation of isobornyl acrylate content in medical devices for diabetes treatment. *Diabetes Technology & Therapeutics*, 21(10), 533-537, 2019.

2.2 Summary of the two publications

2.2.1 Disrupted biomineralization in zebra mussels after exposure to bisphenol-A: Potential implications for molar-incisor hypomineralization

Over the past few decades, thousands of studies have investigated the aetiology of MIH which is considered as multifactorial, such as early childhood diseases (e.g., high fever), environmental toxicant exposure (e.g., dioxins), antibiotic use (e.g., amoxicillin, erythromycin), hypoxia, and genetic variants associated with enamel formation [4, 5, 16, 17]. However, the evidence level for aetiology of MIH remains low, because many of the existing studies are retrospective and small sample sizes [4, 5]. Experimental studies reported that enamel defects were caused by acute hypoxia, amoxicillin, bisphenol-A (BPA) in rodents [18-23]. Nevertheless, the German Federal Institute for Risk Assessment evaluated the study of BPA as not being fully qualified because it was investigated only on male rats and one dose of BPA [24]. Furthermore, with rodents, only incisors have enamel and keep growing throughout life [25]. Additionally, ameloblasts (mineralization organ of enamel) degenerate or apoptosis after enamel eruption [26, 27].

Teeth enamel is the hardest tissue in humans as it is composed of >95% of minerals (hydroxyapatite) and <5% of organic matrix (proteins, polycarbonate, etc.) which is similar with that of shell in mollusk [28, 29]. Moreover, both mineralization of teeth enamel shells is secreted and regulated by epithelial cells [26, 30]. In the last decades, invertebrate models have been evaluated and considered as valid models in biomedical research, principally because of their biomineralization of the carbonate shell during growth and ethical restrictions on vertebrate utilization [6, 7]. Mussels are easily exposed to toxins in the water as compared to snails in the air. Cadmium can disrupt the mineralization of both tooth enamel and mussel shells [31, 32]. Therefore, zebra mussels might be a potential animal model for biomineralization.

BPA, as the constituent of epoxy resins and polycarbonate plastics, can migrate out of plastic baby feeding bottles [33]. Because of its structural similarity to the estrogen 17β-estradiol,

BPA is an endocrine-disruptive chemical linked to dysfunction in the immune system and other physical functions involving multiple endocrine-related pathways [34, 35]. BPA induced enamel hypomineralization in rats by disturbing signalling pathways of oestrogen and androgen [23]. Furthermore, MIH is linked to the intake of erythromycin in the 1st year of life [21]. Calcein generates sustained fluorescence by binding to calcium ions that is involved in the formation of calcium carbonate crystals in hard tissues, and thus has been frequently applied as a marker for shell growth [7, 36].

Study design The registration of experiments on zebra mussels is non-essential according to the German Animal Protection Regulation. Zebra mussels (*Dreissena polymorpha*) were acquired from the Schinderbach River (Danube catchment, Bavaria, Germany). The mussels were kept in well-aerated tap water at 15-20°C until the experiemnts. Zebra mussels (shell length, 1.9 ± 0.2 cm) were divided into 46 groups (n = 7 mussels/group) for four experiments (12 groups/experiment, BPA and doxycycline experiments shared the two control groups) linked to four substances including cadmium (positive control), BPA, erythromycin, and doxycycline (no association with MIH and a calcium ions chelator with fluorescence). All solutions were mixed with tap water in this study. To label newly mineralized shells, mussels of six groups (per experiment) were incubated with calcein solution (100 mg/l, pH 7.4; Sigma-Aldrich; St. Louis, USA) for 96 h, and another six groups with tap water (controls). As following, mussels both with and without calcein incubation were separately exposed to 0, 0.01, 0.1, 1, 10 and 100 mg/l cadmium sulfate hydrate ($3CdSO_4 \cdot 8H_2O$) (hereafter cadmium), 0, 0.02, 0.2, 2, 20 and 200 mg/l BPA solutions, 0, 0.1, 1, 10, 100 and 1000 mg/l erythromycin and doxycycline solutions for 96 h. Then, shells were collected and stored in 70% (v/v) ethanol after shells growth for two weeks.

Mortality and 96 h-LC50 When a mussel is found to be unresponsive to any stimulus, the shell expands and siphon retracts completely, consider the mussel to be dead [37]. The median lethal concentration at 96 h (96 h-LC50) were calculated using the probit analysis. The mortality of zebra mussels was 100% after 20 and 200 mg/l BPA exposure. Thus, 96 h-LC50 of BPA was 6.3 mg/l (1.3-34.4 mg/l 95% confidence interval (CI)) in zebra mussels. The mortality of zebra mussels was 100% after 10 and 100 mg/l cadmium exposure.

Thereafter, the 96 h-LC50 of cadmium was 3.1 mg/l with 1.6-23.9 mg/l 95% CI in zebra mussels. Additionally, BPA and cadmium were moderate toxic (1.0 < LC50 < 10 mg/l) to zebra mussels.

Histology and quantitative analysis Shells were dehydrated in alcohol solutions, degreased with xylene and methanol, and embedded with methylmethacrylate (MMA). Embedded blocks were cut along the longest growth axis into 400 μm thick sections that were ground to 250 μm thick and polished. The excitation and emission wavelengths of calcein are 494 nm and 517 nm, and that of doxycycline is 370-375 nm and 515 nm [38]. Slides were observed under a confocal microscope (Olympus, BX51WI; Olympus, Tokyo, Japan) with a UPLSAPO10X objective. Photographs with fluorescence were captured by a greyscale EM CCD camera (model C9100-02, 1000×1000 pixels, Hamamatsu Phonetics, Hamamatsu City, Japan) at specific exposure times (maximum intensity value of pixels was less than 255 using the Plot measurement of Stereo Investigator software (version 11.07; MBF Bioscience, Williston, USA). The configuration of the camera was as follows: calcein, exposure time = 15 ms in cadmium and erythromycin experiments, and exposure time = 40 ms in BPA and doxycycline experiments; doxycycline, exposure time = 100 ms; sensitivity = 50 in all measurements. Fluorescence intensity was measured using Stereo Investigator software and summed.

Statistical analysis The differences of fluorescence intensity were analyzed with one way ANOVA-test in SPSS (Version 21.0.1, SPSS Inc., Armonk, NY, USA). Data were log-transformed for ANOVA testing if it is not equal variance or (and) normally distribution. To evaluate interaction and main effects of doxycycline and calcein on doxycycline fluorescence, the univariate ANOVA-test was performed. The level of statistical significance was $p < 0.05$.

Effect of cadmium, BPA, doxycycline, and erythromycin on shells mineralization
Calcein fluorescence decreased after 1 mg/l cadmium exposure ($p < 0.05$) compared with the control, suggesting cadmium disrupted shells biomineralization in zabra mussels. A similar alteration was found in 2 mg/l BPA group, calcein fluorescence decreased compared with the control group ($p < 0.05$), indicating that shells mineralization was inhibited by BPA

in zebra mussels. In the erythromycin and doxycycline experiments, calcein fluorescence were not altered in shells.

Doxycycline fluorescence Doxycycline fluorescence increased significantly ($p < 0.05$) after 0.1, 10, 100 and 1000 mg/l doxycycline exposure, indicating doxycycline can be bind to shells. However, doxycycline fluorescence in shells was not interfered by calcein incubation.

In conclusion, shell mineralization was disturbed by potential MIH-causative chemicals. The present findings demonstrate for the first time that the zebra mussel is a feasible invertebrate model for *in vivo* investigation of causative factors of MIH in humans. Additionally, cadmium and BPA were moderately toxic to zebra mussels. Mussel as a new model achieves the three R's (3R's), to replace and reduce the vertebrate use and to refine hypotheses prior to vertebrate experiments. Thus, it's necessary to identify the effect of vitamin D on BPA-caused hypomineralization. Nevertheless, forthcoming research on the exact interaction mechanisms is necessary to deepen the understanding of the new biomineralization model.

2.2.2 Evaluation of isobornyl acrylate content in medical devices for diabetes treatment

ACD may be caused by substances contained in the adhesive of the medical product or in the plastic material used to house the technical component itself, which implies that a type 4 contact allergic reaction is resulted by allergens in the given medical product and/or in the adhesive. ACD caused by contact allergy to cyanoacrylate used in the glue for fabric parts of glucose sensor set [39]. A positive reaction to hydroxycyanoacrylate in the skin sensitivity patch test from the intermediate adhesive layer in Dexcom G4 kit, 2-ethyl cyanoacrylate was also described as a sensitizer of ACD in Dexcom G4 user [40]. Simillarlly, a patch test with IBOA 0.1% was performed in a boy experiencing ACD induced by wearing Freestyle Libre which showed a positive skin reaction after 48 and 72 hours, whereas no positive skin reactions appeared with the adhesive [41]. IBOA was also detected in the housing of the patch pump Omnipod by gas chromatography (GC) but not in the adhesive [42, 43]. However, both the housing and adhesive were IBOA free in the Dexcom G5 [44].

Sample preparation Different CGM systems was measured as follows: Freestyle Libre [Abbott;], Enlite [sensor LOT D138P, transmitter LOT GT6066295M; Medtronic, Dublin, Ireland] and G6 [LOT 5249363; Dexcom, Inc., San Diego, CA]. CGM systems (Freestyle Libre (n = 3), Enlite sensor (n = 4) and Dexcom G6 (n = 3)) were immersed with 6 ml of methanol containing 10 µg/ml of internal standard caffeine (CF). The samples were incubated for 3 days at room temperature in the dark. The IBOA content in methanol (eluate) was analyzed by gas chromatography-mass spectrometry (GC/MS). The samples were also eluted with water as described previously. Following incubation, the samples were extracted with ethyl acetate (1:1 v/v) for GC/MS analysis. The same technical procedure was conducted on the adhesives pieces of the CGM systems (skin plaster n = 3 and glue layer of the skin adhesive n = 3; Enlite (n = 1) and Dexcom G6 (n = 3).

Analytic procedure The analysis of the eluate was performed on a Finnigan Trace GC super gas chromatograph connected to a DSQ mass spectrometer (Thermo Electron, Dreieich, Germany). For the GC separation, the capillary column is A J&W VF-5ms capillary column (0.25 mm inner diameter, 30 m long; 0.25 lm coating). Helium 5.0 (1 mg/min) was used as the carrier gas. The delivery line was at 250°C. 1 µL of elution was injected with splitless mode (split flow 50 mL/min, splitless time 1 min). An inlet for programmable temperature pores was heated from 30°C to 320°C (14.5°C/s) for capillary transfer and held at this final temperature for 5 min. The GC oven was initially heated isothermally at 50°C for 2 min, then ramped to 280°C (25°C/min) remaining for 5 min. The MS was operated in the electron impact mode at 70eV (ion source temperature: 240°C). Samples were recorded in full scan mode (m/z 50–600). Identification of IBOA was identified by the retention time and mass spectra of the reference standard IBOA (Merck, Darmstadt, Germany) which was callibrated. The limit of quantification (LOQ) of IBOA was 0.1 and 0.2 µg/mL in methanol and water.

No IBOA (< LOQ) was detected in any of the CGM systems (including adhesives) after 3 days eluting with water. For samples with methanol elution of 3 days, IBOA content was 2.64 µg/mL with a standard deviation (SD) of 0.36 µg/mL in Freestyle Libre, 1.11 lg/mL with SD of 0.12 µg/mL in the Enlite CGM system and 0.26 µg /mL its adhesives, whereas no IBOA was detected in Dexcom G6 CGM system and the adhesive.

In conclusion, IBOA can be detected in some CGM system (including adhesives). In view of severity and clinical consequences of allergic skin reactions, manufacturers are encouraged to develop IBOA-free products.

3. Paper I

Fangfang Liu, Franz-Xaver Reichl, Stefan Milz, Uta Christine Wölfle, Jan Kühnisch, Christoph Schmitz, Jürgen Geist, Christof Högg, Katharina Sternecker. Disrupted biomineralization in zebra mussels after exposure to bisphenol-A: Potential implications for molar-incisor hypomineralization. *Dental Materials*, Dental Materials. 2022; 38(4), 689-699.

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Disrupted biomineralization in zebra mussels after exposure to bisphenol-A: Potential implications for molar-incisor hypomineralization

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ARTICLE INFO

ABSTRACT

Objective: The aetiology of molar-incisor hypomineralization (MIH) is currently unclear. A major hurdle in MIH research is the lack of adequate model systems. The study investigated the feasibility of zebra mussel (*Dreissena polymorpha*) as a novel model to screen potential MIH-related factors.

Methods: In four experiments with overall 46 groups (n = 7 mussels/group), six groups per experiment were incubated with 100 mg/l calcein (mineralization marker) solution for 96 h to evaluate the dynamics of shell biomineralization, another six groups with tap water only (controls). Then zebra mussels with and without calcein pre-incubation were exposed to cadmium sulfate hydrate ($3CdSO_4 \cdot 8H_2O$) (positive control; 0, 0.01, 0.1, 1, 10 and 100 mg/l), possible aetiological factors of MIH including bisphenol-A (BPA; 0, 0.02, 0.2, 2, 20 and 200 mg/l) and erythromycin (0, 0.1, 1, 10, 100 and 1000 mg/l) as mineralization "disruptors", and doxycycline (0, 0.1, 1, 10, 100 and 1000 mg/l) for 96 h, respectively. After two weeks, the mussels were sacrificed and shells were embedded in methylmethacrylate for fluorescence intensity analysis.

Results: Mortality rate was 100% after 20, 200 mg/l BPA and 10, 100 mg/l $3CdSO_4 \cdot 8H_2O$ exposure. Thereby, the median lethal concentration (96 h-LC50) of BPA was 6.3 mg/l (95% CI, 1.3–34.4 mg/l), and that of cadmium was 3.1 mg/l (95% CI, 0.7–10.5 mg/l). Notably, calcein fluorescence in shells significantly decreased ($p < 0.05$) after 2 mg/l BPA and 1 mg/l $3CdSO_4 \cdot 8H_2O$ exposure.

Significance: These findings suggest that BPA may disrupt biomineralization. Biomineralization in zebra mussels seems to be an effective model for investigating potential MIH-related factors.

1. Introduction

Molar-incisor hypomineralization (MIH) is a prevalent developmental defect of the enamel that affects primary and permanent teeth, most frequently the first permanent molars and incisors [1]. Hypomineralization can be detected clinically as demarcated opacities in enamel that vary in color and size, indicating that the ameloblast-controlled mineralization process was disturbed in the (early) maturation stage [2]. The global prevalence of MIH is relatively high but varies significantly across countries and regions [3,4]. Overall, MIH is considered a global health problem and a daunting dental health challenge [5]. While the epidemiological situation is well described, the aetiology of MIH remains unclear and is considered to be multifactorial. Early childhood illnesses (particularly high fever), antibiotic use (amoxicillin and erythromycin), hypoxia, hypocalcaemia, environmental toxicant exposure and genetic variations in enamel formation-related genes have been discussed as possible causes of this disease [6–9]. Many existing studies were performed retrospectively by collecting information using questionnaires dependent on parents´ memory, and potential confounders were not adjusted in the majority of these studies; moreover, the number of participants was usually small; thus, the evidence level for possible causative factors remains low; in addition, it is difficult to compare outcomes among studies due to the use of different exposure criteria [6,7]. Experimental studies have been performed in different vertebrates, and different laboratories identified divergent factors. For example, severe acute hypoxia caused enamel defects in mice [10]. However, the high incidence of MIH in children cannot be explained by severe acute hypoxia [11]. Amoxicillin exposure was associated with hypomineralized enamel in rats, but hypomineralization was not clinically detectable in pigs [12–14]. Bisphenol-A (BPA) caused enamel defects in rats by disrupting normal protein removal during amelogenesis [15]. However, in rodents, only incisors have enamel, and they also lack a root structure and grow continuously throughout life, reflecting molecular and cellular differences between human and rat teeth [16,17]. It is not possible to determine the aetiology and mechanisms of MIH in vitro because ameloblasts are lost after enamel eruption; furthermore, the potentially relevant organic matrix from early developmental stages does not exist in mature enamel tissue [18,19]. With regard to animal models, pigs (in contrast to rodents) are a common model in dentistry because their maxillofacial and oral structures are comparable to those of humans in terms of physiology, anatomy and disease development [20]. However, research on pigs is expensive and limited by ethical and husbandry-related constraints, which renders pigs unsuitable for screening potential aetiological factors of MIH. Thus, there is an urgent need for a novel, inexpensive and rapid animal model that is free of the ethical constraints associated with vertebrate experimentation and can be used to screen potential aetiological factors of MIH and other biomineralization-related conditions in vivo [21].
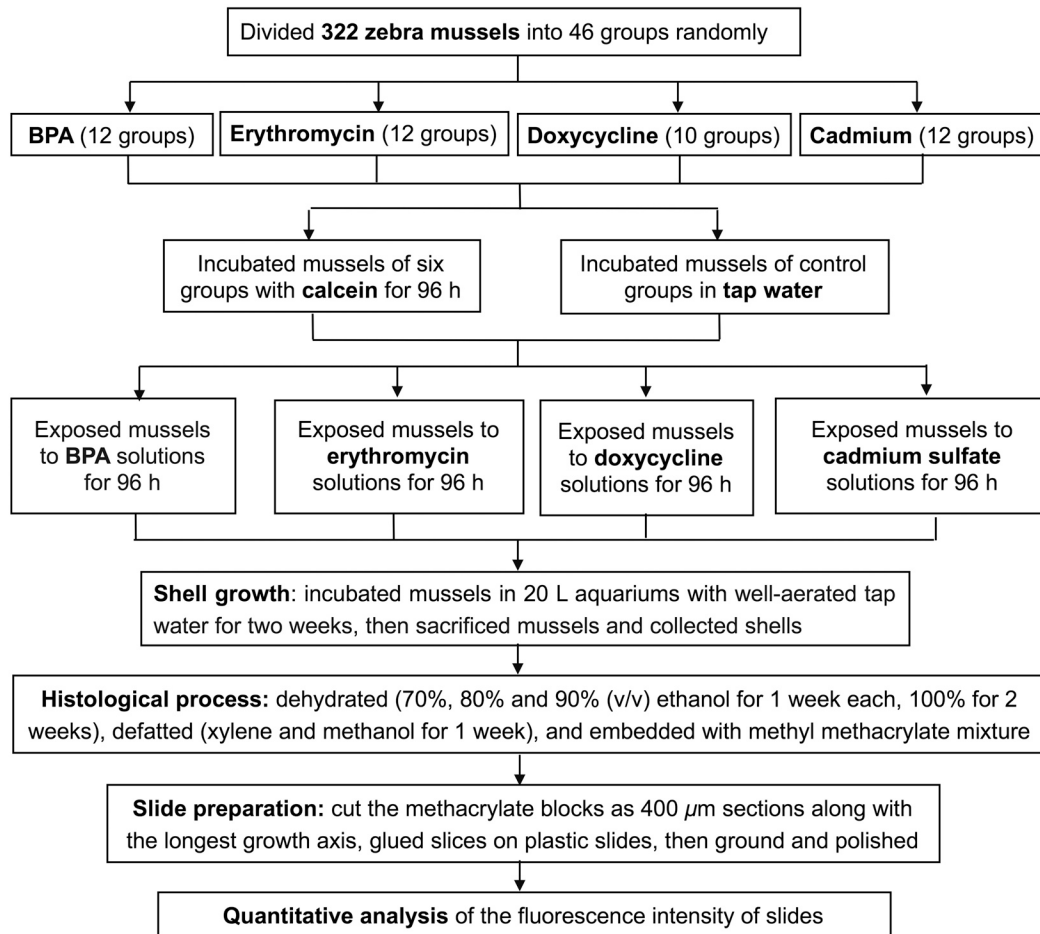
In recent decades, zebra mussels have been identified as a useful model for studying biomineralization because there are many similarities between the basic mineralization processes of enamel (teeth) and nacre (mussels). Both biomineralization processes start with organic matrix framework assembling, followed by forming of primary minerals, nucleation and growth of crystals, which is strictly controlled by epithelium cells [22,23]. BPA is an important and widely used component of polycarbonate plastics (e.g., used in reusable food and drink containers, medical equipment, electronic equipment and sports safety equipment) and epoxy resins (e.g., used in internal protective coatings for food and beverage containers, electronic laminates, paints and adhesives) (http://www.bisphenol-a.org/about/index.html). The migration of BPA from plastic baby bottles (even from those with a BPA-free/safe label) was detected in high-performance liquid chromatography tests, and this release increased significantly as the cycles of brushing and washing with detergent increased [24,25]. Calcein green (hereafter calcein), a fluorescent indicator that binds to mineral deposition sites, has been used repeatedly as a marker of shell growth in bivalvia because it produces long-lasting fluorescence by interacting with the calcium ions that participate in the formation of calcium carbonate crystals in hard tissues [26,27].

In the present study, the zebra mussel model was applied to investigate the effects of potential MIH-causative factors on biomineralization. To this end, zebra mussels (*Dreissena polymorpha*) were exposed to two substances that are known as mineralization disruptors and have been reported to cause MIH, namely, BPA and erythromycin. To validate the results, control groups were exposed to the antibiotic doxycycline, a known chelation-inducing agent and a fluorescent marker of newly mineralized tissues [28], as well as to cadmium sulfate hydrate $(3CdSO_4 \bullet 8H_2O)$ (hereafter cadmium), which is known to inhibit the mineralization of both zebra mussel shells and tooth enamel [29–32].

2. Materials and methods

All experiments followed German Animal Protection Regulations, which do not require the registration of experiments with zebra mussels. The study design is shown in Fig. 1a. The study is reported following the ARRIVE guidelines.

a

Divided **322 zebra mussels** into 46 groups randomly

BPA (12 groups) **Erythromycin** (12 groups) **Doxycycline** (10 groups) **Cadmium** (12 groups)

Incubated mussels of six groups with **calcein** for 96 h

Incubated mussels of control groups in **tap water**

Exposed mussels to **BPA** solutions for 96 h

Exposed mussels to **erythromycin** solutions for 96 h

Exposed mussels to **doxycycline** solutions for 96 h

Exposed mussels to **cadmium sulfate** solutions for 96 h

Shell growth: incubated mussels in 20 L aquariums with well-aerated tap water for two weeks, then sacrificed mussels and collected shells

Histological process: dehydrated (70%, 80% and 90% (v/v) ethanol for 1 week each, 100% for 2 weeks), defatted (xylene and methanol for 1 week), and embedded with methyl methacrylate mixture

Slide preparation: cut the methacrylate blocks as 400 μm sections along with the longest growth axis, glued slices on plastic slides, then ground and polished

Quantitative analysis of the fluorescence intensity of slides

b

Groups	BPA (mg/l)	Erythromycin (mg/l)	Doxycycline (mg/l)	$3CdSO_4 \bullet 8H_2O$ (mg/l)
A/CA	0	0	0	0
B/CB	0.02	0.1	0.1	0.01
C/CC	0.2	1	1	0.1
D/CD	2	10	10	1
E/CE	20	100	100	10
F/CF	200	1000	1000	100

Fig. 1 – Flow chart of the study design and protocol. a) A total of 322 zebra mussels were randomly divided into 46 groups (n = 7 mussels/group, bisphenol-A (BPA) and doxycycline experiments shared the same control groups) for four experiments (12 groups/experiment) to investigate potential causative factors of MIH by assessing the formation of mineralized tissues after BPA and erythromycin (potential causative factors of MIH) as mineralization "disruptors", doxycycline (as a chelation-inducing agent, but not associated with MIH), and cadmium sulfate hydrate ($3CdSO_4 \bullet 8H_2O$, positive control) exposure after calcein green (mineralization marker) incubation. b) Exposures of the different groups. The CA, CB, CC, CD, CE, and CF groups were the groups incubated with 100 mg/l calcein, and the A–F groups were the sham control groups.

2.1. Animals

Colonies of zebra mussels (*Dreissena polymorpha*) were collected from uncontaminated sites in the Schinderbach River (Danube catchment, Bavaria, Germany) in November 2018 and June 2019. The mussels were kept in a 50 L glass aquarium containing well-aerated tap water at 15–20 °C until the experiment and fed *Chlorella vulgaris* (SAG Number 211-19; Algae collection of the University of Goettingen, Germany) twice a week *ad libitum*. To investigate the effects of BPA, erythromycin, doxycycline and cadmium (positive control) exposure on shell mineralization in zebra mussels, four experiments were performed in this study. A total of 322 zebra mussels were picked from a pool of healthy mussels (maximum shell length, 1.9 ± 0.2 cm) and randomly divided into 46 groups (n = 7 mussels/group) for four experiments (12 groups/experiment, BPA and doxycycline experiments shared the same control groups) [33]. All solutions were mixed with tap water in this study. To label newly mineralized tissues, the mussels in six groups (per experiment) were incubated in 200 ml of calcein solution (100 mg/l, pH 7.4; Sigma-Aldrich; St. Louis, USA) for 96 h, and the mussels in six groups were incubated with tap water (controls).

2.2. Mussel exposure

Five different concentrations of BPA, erythromycin, doxycycline and cadmium were used in this study. From zero to the highest concentration, we set the non-calcein groups as the A–F groups, and calcein incubated groups as the CA, CB, CC, CD, CE, and CF groups in the four experiments (Fig. 1b). The highest concentration of BPA for exposure was 200 mg/l in this study because the solubility of BPA in water is 288 mg/l at 25 °C [34]. For the 200 mg/l BPA solution, 500 ml BPA (Sigma-Aldrich) solution was mixed and then subjected to ultrasonic treatment for 4 h and mechanical vibration overnight for complete dissolution. Other BPA solutions were diluted from the 200 mg/l BPA solution. Mussels were exposed to 200 ml of 0 (A, CA), 0.02 (B, CB), 0.2 (C, CC), 2 (D, CD), 20 (E, CE) and 200 mg/l (F, CF) BPA solutions for 96 h. For the experiment involving antibiotics, mussels were exposed to 0 (A, CA), 0.1 (B, CB), 1 (C, CC), 10 (D, CD), 100 (E, CE) and 1000 mg/l (F, CF) erythromycin (Inresa Arzneimittel GmbH, Germany) solutions for 96 h. Additionally, to assess effects on shell mineralization and labeling of newly mineralized tissues, mussels were exposed to 0 (A, CA), 0.1 (B, CB), 1 (C, CC), 10 (D, CD), 100 (E, CE) and 1000 mg/l (F, CF) doxycycline (Sigma-Aldrich) solutions for 96 h. In this study, 100 mg/l was the highest concentration of cadmium solution (Sigma-Aldrich) used. Mussels were exposed to 0 (A, CA), 0.01 (B, CB), 0.1 (C, CC), 1 (D, CD), 10 (E, CE) and 100 (F, CF) mg/l cadmium solutions for 96 h. Mussels were exposed with BPA and doxycycline in January 2019 (winter) as well erythromycin and cadmium in June and July 2019 (summer), separately.

Following BPA, erythromycin, doxycycline and cadmium exposure, the mussels were incubated in 20 L aquariums containing well-aerated tap water (water temperature 21 ± 1.03 °C, dissolved oxygen 7.78 ± 0.72 mg/l, pH 7.95 ± 0.47) to form new mineralized tissues. Shells of living mussels were dissected after growing for two weeks and stored in 70% (v/v) ethanol for histologic analysis.

2.3. Mortality rates and 96 h-LC50

Zebra mussels were checked daily for survival, and dead mussels were removed each day. Mussels were considered dead when no response was noted to any stimuli, the shell gape was wide, and the siphons were totally retracted [35]. The mortality rate was expressed as the ratio of the number of dead mussels after BPA, erythromycin, doxycycline and cadmium sulfate exposure to the original total number of mussels. The median lethal concentration at 96 h (96 h-LC50) and the 95% confidence interval (CI) were calculated using the probit analysis statistical method.

2.4. Histology

Shells were dehydrated in alcohol solutions of increasing concentration (70%, 80%, and 90% (v/v) ethanol for one week and then 100% ethanol for two weeks). Then, the shells were immersed in both xylene and methanol for one week for degreasing and embedded with methylmethacrylate (MMA) as described in the study of Milz et al. [36]. After polymerization, MMA blocks were cut into 400 μm thick sections along the longest growth axis using a Leica SP 1600 saw microtome (Leica, Wetzlar, Germany). Finally, the sections were ground to 250 μm thick and polished with a 400 CS grinder (EXAKT Advanced Technologies, Norderstedt, Germany).

2.5. Quantitative analysis

The excitation wavelength of calcein is 494 nm, and the emission wavelength is 517 nm; the excitation wavelength of doxycycline is 370–375 nm, and the emission wavelength is 515 nm [37,38]. Slides labeled with calcein or doxycycline were observed under a confocal microscope (Olympus, BX51WI; Olympus, Tokyo, Japan) with a UPLSAPO10X objective, an Alexa Fluor 488 filter (excitation: 498 nm, emission: 502 nm for calcein fluorescence imaging) and an Alexa Fluor 350 filter (excitation: 346 nm, emission: 442 nm for doxycycline fluorescence imaging). Photographs of the fluorescence of newly mineralized tissues were taken by a greyscale EM CCD camera (model C9100-02, 1000 × 1000 pixels, Hamamatsu Phonetics, Hamamatsu City, Japan) with a SOLA LED lamp (Lumencor, Beaverton, USA) at appropriate exposure times when the highest intensity value of pixels was less than 255 (maximum intensity at 8 bit resolution) using the Plot measurement of the software Stereo Investigator (version 11.07; MBF Bioscience, Williston, USA). Fluorescence intensities were adjusted by specifying different exposure times to eliminate also the effects of potential different seasonal growth in summer or winter or additional effects e.g. of the fluorescence of the doxycycline. The configuration of the camera was as follows: calcein, exposure time = 15 ms, sensitivity = 0 and gamma = 1.0 in cadmium and erythromycin experiments, and exposure time = 40 ms, sensitivity = 0 and gamma = 1.0 in BPA and doxycycline experiments; doxycycline, exposure time = 100 ms, sensitivity = 50 and gamma = 1.0. The diagram of the whole experimental process and
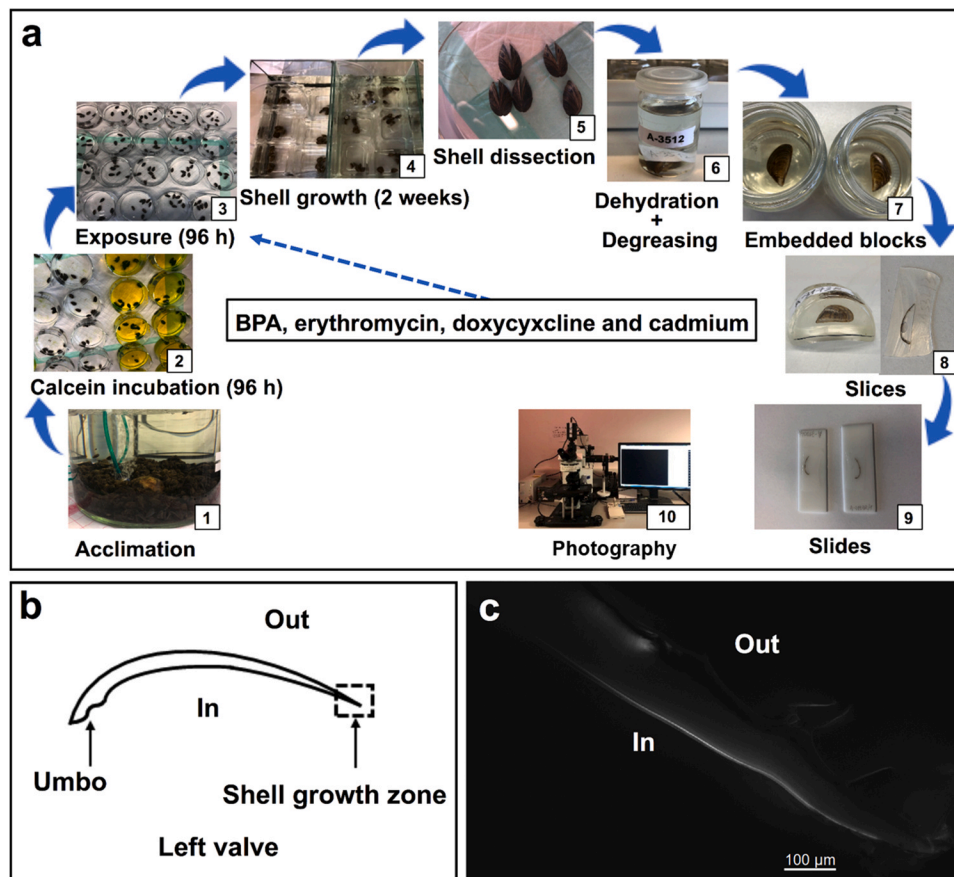
Fig. 2 – a) Flow diagram of the experimental process; b) schematic of a section of a mussel left valve and the square marked shell growth zone; c) fluorescence signal of calcein in the shell growth zone of zebra mussels.

photos of the fluorescence of shells are shown in Fig. 2. The process of plot measurement is shown in Fig. 3. The fluorescence intensity signal was measured with Stereo Investigator software, and the sum of the fluorescence intensities was calculated.

2.6. Statistical analysis

Descriptive and explorative analyses were performed using SPSS (Version 21.0.1, SPSS Inc., Armonk, NY, USA). The sum of fluorescence intensity in newly mineralized tissues was expressed as the mean ± standard error of the mean (SEM). D'Agostino-pearson test and Levene's test were performed to test the normality and the equality of variances of the data. Data were log-transformed when it's not normally distribution or (and) unequal variances before ANOVA-test. The differences in fluorescence intensity of calcein were analyzed using one way ANOVA-test in SPSS. The univariate ANOVA-test for factorial designs was performed to evaluate each main effect and interaction of calcein and doxycycline on doxycycline fluorescence. Dunnett's t test was performed to evaluate the significance of difference with the control groups. $p < 0.05$ was set as the level of statistical significance.

3. Results

3.1. Mortality rate and 96 h-LC50

No differences in the mortality rate were observed between the groups incubated to calcein green and the non-calcein groups subjected to sham incubation for 96 h before exposure to BPA, erythromycin, doxycycline or cadmium. The mortality rate of the zebra mussels was 100% in the E and CE groups (20 mg/l) and the F and CF groups (200 mg/l) after BPA exposure (Fig. 4a). Accordingly, the median lethal concentration at 96 h (96 h-LC50) for BPA was 6.3 mg/l, with a 1.3–34.4 mg/l 95% confidence interval (CI) in zebra mussels. For antibiotics, the mortality rate of zebra mussels was 14.3% in both the F and CF groups (1000 mg/l) after erythromycin exposure (Fig. 4b). The mortality rate was 28.6% in the CF group (1000 mg/l) in the doxycycline experiment and 71.4% in the F group (Fig. 4c). Additionally, the mortality rate of zebra mussels was 100% in the E (10 mg/l) and F (100 mg/l) groups after cadmium exposure (Fig. 4d). Therefore, the 96 h-LC50 of cadmium was 3.1 mg/l (95% CI, 1.6–23.9 mg/l) in zebra mussels. After cadmium exposure, the mortality rates of the zebra mussels were 14.3%, 85.7%, and 100% in the CD (1 mg/l), CE (10 mg/l), and CF (100 mg/l) groups (Fig. 4d).
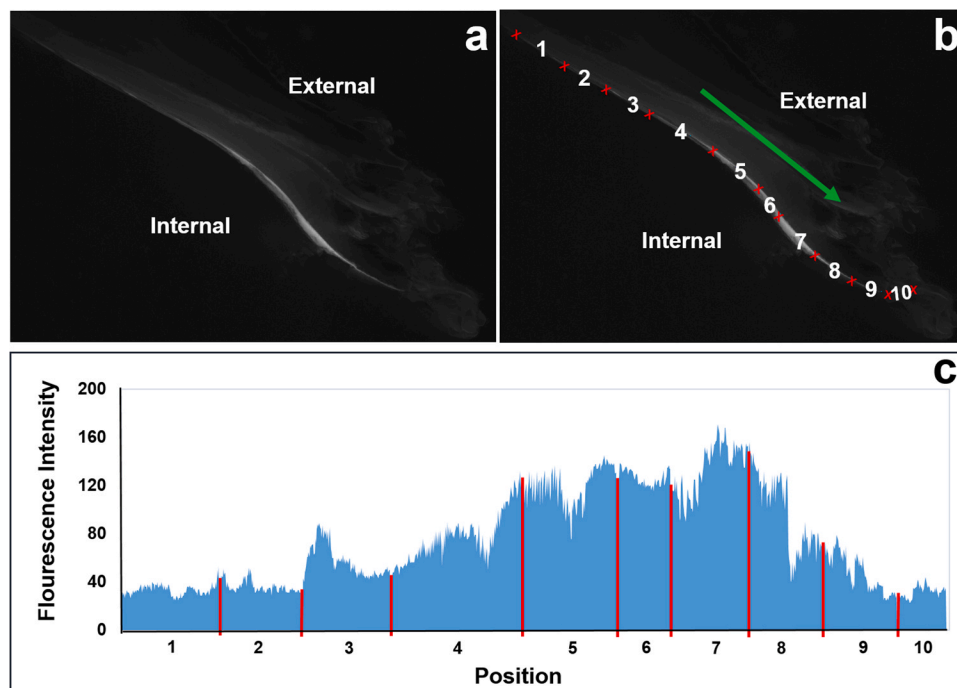
Fig. 3 – Measurement of the fluorescence intensity in the shell growth zone. a) The shell growth zone showing the fluorescence signal. b) Red crosses mark the hypostracum as several sections (representing as 1–10 linear pixel plots in this sample) in the curved growth zone to measure the fluorescence intensity continuously; the green arrow indicates the direction of the linear pixel plots. c) Representative linear pixel plot of the fluorescence intensity in the shell growth zone used for analysis; red lines mark the sections of the linear pixel plots shown in b.

3.2. Fluorescence intensity of calcein

To investigate the effects of BPA, erythromycin, doxycycline and cadmium on shell mineralization in zebra mussels, the fluorescence intensity of calcein in newly mineralized tissues was evaluated. Broken shells caused by technical issues in the histology process and shells with no fluorescence signal were excluded from the analysis. In the cadmium experiment, a non-calcein fluorescence signal was observed in one and two shells from the CC and CA groups. In the BPA experiment, one shell was broken in the CA group; a non-calcein fluorescence signal was observed in one shell from the CA and CB groups and in two shells from the CC group. One shell was broken in the CA and CC groups in the erythromycin experiment; a non-calcein fluorescence signal was observed in one shell from the CD and CE groups. In the doxycycline experiment, one shell was broken in the CA group; a non-calcein fluorescence signal was observed in one shell from the CA, CB and CE groups.

The strongest calcein fluorescence was found in shells from the CA group; calcein fluorescence decreased in a concentration-dependent manner after cadmium exposure ($p < 0.05$) (Fig. 5a). Moreover, the calcein fluorescence of the shells was significantly different ($p = 0.0071$) between the CD group (1 mg/l) and the CA group in the cadmium experiment (Fig. 5a). A similar alteration was found in the BPA experiment, i.e., the strongest calcein fluorescence was found in shells from the CA group, and the weakest calcein fluorescence was found in shells from the CD group (2 mg/l)

($p < 0.05$) (Fig. 5b). Furthermore, compared with that of shells from the CA group, calcein fluorescence significantly decreased ($p < 0.05$) in shells from the CD group (2 mg/l) after BPA exposure. In the erythromycin experiment, the strongest calcein fluorescence was found in shells from the CD group (10 mg/l), and the weakest calcein fluorescence was found in shells from the CE group (100 mg/l) (Fig. 5c). After doxycycline exposure, the strongest calcein fluorescence was found in shells from the CD group (10 mg/l), and the weakest calcein fluorescence was found in shells from the CE group (100 mg/l) (Fig. 5d). However, calcein fluorescence was not altered after erythromycin and doxycycline exposure (Figs. 5c and 5d).

3.3. Fluorescence intensity of doxycycline

In the doxycycline experiment, one shell was broken in the A, CA, CB, and CE groups; a non-calcein fluorescence signal was observed in one shell from the CB, CE, F and CF groups, and two shells from the D group. Doxycycline fluorescence in shells was assessed to explore its ability to label newly formed mineralized tissues. Main effect tests showed significant change of doxycycline fluorescence in shells after different concentrations doxycycline exposure ($p < 0.05$) (Table 1). The strongest doxycycline fluorescence was found in shells from the CD group (10 mg/l), and the weakest doxycycline fluorescence was found in shells from the CA group. Furthermore, significantly increased ($p < 0.05$) doxycycline fluorescence occurred after 0.1, 10, 100, and 1000 mg/l doxycycline exposure compared with the sham control.
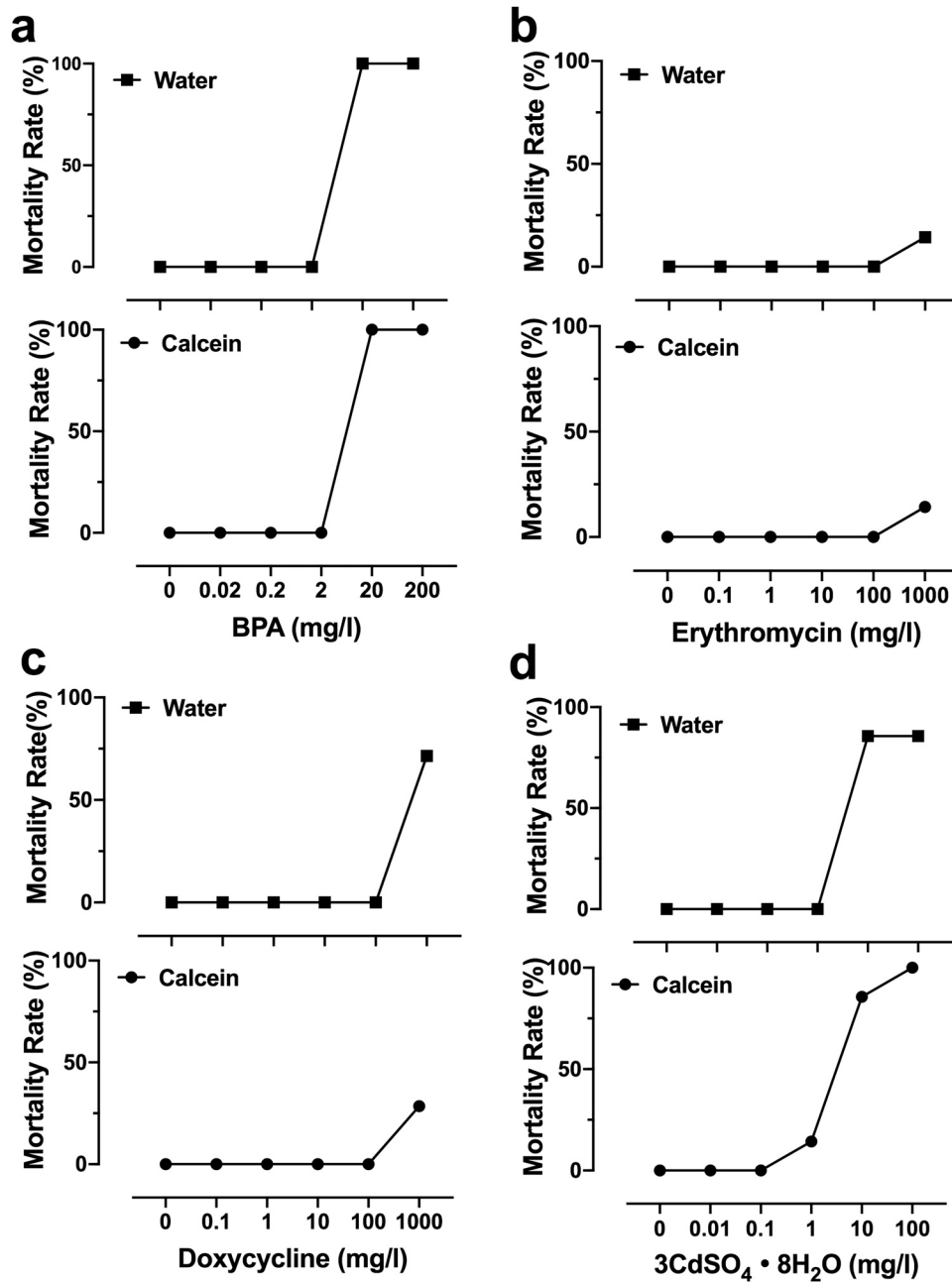
Fig. 4 – Mortality of zebra mussels after exposure to a) 0, 0.02, 0.2, 2, 20 and 200 mg/l BPA solutions in the calcein-incubated (100 mg/l) and sham control groups; b) 0, 0.1, 1, 10, 100 and 1000 mg/l erythromycin solutions in the calcein-incubated and sham control groups; c) 0, 0.1, 1, 10, 100 and 1000 mg/l doxycycline solutions in the calcein-incubated and sham control groups; and d) 0, 0.01, 0.1, 1, 10 and 100 mg/l 3CdSO$_4$•8H$_2$O solutions in the calcein-incubated and sham control groups.

However, no change of doxycycline fluorescence in shells was found with or without the initial incubation with calcein. Furthermore, no interaction effect was found for doxycycline fluorescence in shells for calcein and doxycycline.

4. Discussion

In the present study, exposure of zebra mussels to BPA and cadmium, but not to erythromycin and doxycycline disrupted shell biomineralization in mussels. These results demonstrate, for the first time, that zebra mussels are a feasible invertebrate model for in vivo investigations of the aetiology of MIH in humans.

In recent decades, invertebrate models have been evaluated and considered effective models in biomedical research, mainly due to ethical concerns regarding the use of vertebrates [21]. Recently, mussels were used as target organisms because their carbonate shells undergo biomineralization during growth [27]. In addition to lower ethical
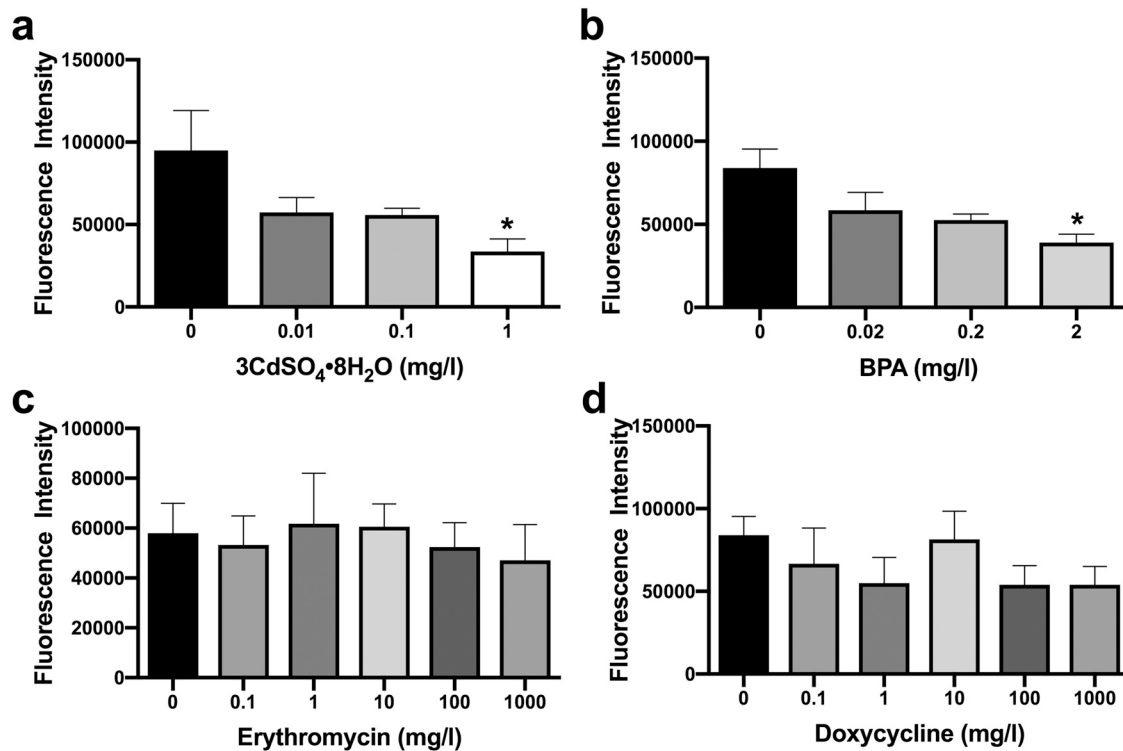
Fig. 5 – The fluorescence intensity of calcein in mussel shells. The panel shows the mean and standard error of the mean of the total fluorescence intensity in calcein fluorescence imaging after zebra mussels were exposed to a) 0, 0.01, 0.1, and 1 mg/l $3CdSO_4 \cdot 8H_2O$ solutions (n = 5, 7, 6, and 6, respectively); b) 0, 0.02, 0.2, and 2 mg/l BPA solutions (n = 5, 6, 5, and 7, respectively); c) 0, 0.1, 1, 10, 100, and 1000 mg/l erythromycin solutions (n = 6, 7, 6, 6, 6 and 6, respectively); and d) 0, 0.1, 1, 10, 100 and 1000 mg/l doxycycline solutions with preincubation with 100 mg/l calcein (n = 5, 6, 7, 7, 6, and 5, respectively). *Significantly different ($p < 0.05$) from the negative control.

concerns compared to the use of vertebrates, zebra mussels can occur at very high density, with high reproduction rates and 6000–30,000 larvae produced by one female mussel [39]. Moreover, the maximum shell length of zebra mussels can reach 35–40 mm, with a growth rate of 15–20 mm/year [39]. Mussel shells consist of three layers comprising periostracum, prismatic and nacreous layers (from outside to inside) where the newly formed mineral phase deposits [40,41]. Additionally, both mature tooth enamel and nacre are composed of approximately 95% mineral with an organic matrix, although the mineral type is hydroxyapatite in tooth enamel and aragonite in the nacre [41,42]. On the other hand, both the hierarchical structure and mineralization of nacre are similar to those of tooth enamel, and they have superior mechanical properties in terms of fracture resistance, high stiffness and toughness [43]. Furthermore, outer mantle epithelial cells are responsible for the formation of nacre in mussels [44]. Of note, tooth enamel is the only epithelium-

Table 1 – Main effects of doxycycline and calcein on doxycycline fluorescence in mussel shells (mean ± SEM).					
Doxycycline (mg/l)	Calcein (mg/l)		Total	Main effect	
	0	100		F	p
0	17727 ± 3891 (n = 6)	14884 ± 5262 (n = 6)	16306 ± 3149 (n = 12)	4.42	0.002
0.1	27575 ± 6360 (n = 7)	37138 ± 9320 (n = 5)	31560 ± 5296* (n = 12)		
1	20406 ± 5302 (n = 7)	30737 ± 4931 (n = 6)	25174 ± 3802 (n = 13)		
10	53949 ± 21636 (n = 5)	57902 ± 12438 (n = 7)	56077 ± 11489* (n = 12)		
100	37120 ± 5759 (n = 7)	43234 ± 8955 (n = 5)	39667 ± 4851* (n = 12)		
1000	17012 (n = 1)	46284 ± 13047 (n = 4)	40430 ± 11679* (n = 5)		
Total	30670 ± 4655 (n = 33)	38365 ± 4368 (n = 33)	34460 ± 3206 (n = 66)		
Main effect F	2.66			NS	
p	0.11				
NS, no intersection between doxycycline and calcein on doxycycline fluorescence.					
* Significantly different (p < 0.05) from the control group.					

derived biomineral in mammals because ameloblasts, the enamel organ, are derived from inner dental epithelial cells [19]. Moreover, the basic mineralization processes of enamel and nacre are comparable. Specifically, inner dental epithelial cells differentiate into ameloblasts that synthesize and secrete a large amount of enamel matrix proteins (EMPs) into the enamel space, and ribbon-like crystals form to generate the full thickness of the enamel layer [22]. Then, these crystals grow in width and thickness by ions deposition, resulting in enamel hardening with EMP degradation and removal [19,45]. Similar to amelogenesis, the nacre tablet formation model is built with an organic framework (a chitin layer that facilitates acidic protein absorption) filled with silk-like proteins [46]. The outer mantle epithelial cells synthesize and secrete ingredients for shell calcification into the extrapallial space (the gap between the mantle epithelium and the shell), along with the formation of amorphous calcium carbonates (the first mineral formation phase), which finally transform into aragonite tablets [23,44,47,48]. Therefore, zebra mussels (*Dreissena polymorpha*) were identified as a promising model for studying biomineralization [41].

In aquatic toxicology, the toxicity categories of substances are as follows: very toxic (LC50 < 1.0 mg/l), moderately toxic (1.0 mg/l ≤ LC50 ≤ 10 mg/l), and less toxic (LC50 > 10 mg/l) [49]. The order of toxicity to zebra mussels was ranked as follows in this study: cadmium > BPA > erythromycin and doxycycline. Cadmium is toxic to zebra mussels and freshwater bivalves (*Anodonta cygnea*) [31,32]. The 96 h-LC50 of cadmium was highest in zebra mussels, with a concentration of 3.1 mg/l (95% CI, 1.6–23.9 mg/l). Moreover, BPA was moderately toxic to fish and invertebrates [50]. BPA was moderately toxic (96 h-LC50, 6.3 mg/l, with a 95% CI of 1.3–34.4 mg/l) in zebra mussels in the present study. The mortality rates of zebra mussels were lowest during exposure, with the highest concentrations (1000 mg/l) of erythromycin (14.3%) and doxycycline (28.6%). This is not surprising, as both substances are used as antibiotics in humans and thus have to present low toxicity values.

As a competitor of essential metal ions, cadmium compounds can disrupt the biomineralization of hard tissues by interfering with the function of essential metal ions and cellular functions related to biomineralization [51]. Hydroxyapatite crystal growth in incisors was disordered after cadmium exposure in rats with significantly increased cadmium levels [29,30]. For invertebrates, shell biomineralization was disturbed by cadmium ions in zebra mussels and freshwater bivalves (*Anodonta cygnea*) [31,32]. The related mechanism might be that cadmium accumulates and is eliminated in the mantle, i.e., the tissue that surrounds the mineralized shell and is responsible for biomineralization in mussels [52]. Therefore, cadmium exposure was used as the positive control in the present study. Calcein fluorescence significantly ($p < 0.05$) decreased in the shells of zebra mussels after exposure to 1.0 mg/l cadmium in the present study. These results confirmed that cadmium inhibited the biomineralization of shells in zebra mussels.

Oral ingestion from foods is the main route for BPA exposure in humans [53]. In addition, the highest estimated BPA dietary exposure (2.0–2.4 µg/kg body weight per day) for infants 0–6 months of age occurred in infants who were exclusively fed canned liquid infant formula using polycarbonate bottles [54]. Enamel formation usually takes place between prenatal day 118 (4 months) and postnatal day 321 (11 months) [42]. Due to its similar structure to the natural oestrogen 17 *β*-oestradiol, BPA is an endocrine-disrupting chemical related to disorders of the immune system and other body functions involving multiple endocrine-related pathways because of its high affinity towards oestrogen receptors [55,56]. Another study reported that BPA caused enamel hypomineralization in rats by disrupting oestrogen and androgen signaling pathways, thus BPA might be a causative factor of MIH in humans [15]. However, the German Federal Institute for Risk Assessment assessed the latter study as not entirely fulfilled because only male rats and only one dose of BPA were investigated (and, thus, no dose-response relationship was established) [57]. Additionally, BPA caused genotoxicity and disruption of the endocrine and immune systems in blue mussels (*Mytilus galloprovincialis*) and green mussels (*Perna viridis*) because BPA stimulated kinase-mediated cell signaling in mantle tissues [58,59]. In the present study, the calcein fluorescence in shells significantly ($p < 0.05$) decreased after exposure of zebra mussels to 2 mg/l BPA. This is in line with the results of another study that found that exposure of blue mussels (*Mytilus galloprovincialis*) in the early life stage to BPA caused irregularities in shell formation, with downregulation of biomineralization-related genes (extrapallial protein and carbonic anhydrase) [60]. In summary, there is ample evidence suggesting that BPA could be a potential causative factor of enamel hypomineralization in MIH in humans.

As a macrolide, erythromycin is often used to treat ear infections or chest infections in children. (https://www.nhs.uk/medicines/erythromycin/) MIH in children was reported to be associated with erythromycin intake in the first year of life [61]. In mice, enamel hypomineralization induced by erythromycin was shown to be related to enamel thickness instead of calcium and phosphate content, with downregulation of inducible enzymes such as cyclooxygenase 2 [62]. With regard to invertebrates, erythromycin caused DNA damage in haemocytes in blue mussels (*Mytilus edulis*), whereas the production of intracellular reactive oxygen species was suppressed, with decreased phagocytosis [63]. In addition, antioxidant defences were induced by erythromycin in the gills of blue mussels (*Mytilus edulis*), while metabolic balance may be disrupted at high concentrations [64]. However, in the present study, calcein fluorescence was not altered in shells after exposure of zebra mussels to erythromycin. A possible explanation might be that the selected concentrations of erythromycin were too low to inhibit shell biomineralization in the present study.

Doxycycline is one of the most popular tetracycline derivatives currently available. An association was reported between tooth staining and doxycycline use in young children [65]. Calcein fluorescence exhibited no alteration in shells after doxycycline exposure in our study. Similarly, doxycycline fluorescence was not effected by calcein incubation. Additionally, doxycycline fluorescence was significantly increased ($p < 0.05$) after 0.1, 10, 100, and 1000 mg/l doxycycline exposure, indicating that doxycycline was incorporated into shells.

In essence, the findings of the present study suggest that the proposed model zebra mussel is a fast and effective in vivo biomineralization model at fairly low cost, with low ethical issues and applications beyond MIH research, and the establishment of comparable dose-response relationships. The main limitation of the present study is the fact that results from mussels cannot be directly applied to humans, so further research on transferability is necessary. Additionally, calcein at higher doses could inhibit mineralization processes owing to its high calcium affinity, which may slightly disorder the real effects of chemical substances on biomineralization [34]. Nevertheless, in our opinion, the presented biological model constitutes an excellent basis for a novel screening system for chemicals interfering with biomineralization.

In conclusion, potential MIH-causative chemicals disrupted shell mineralization, suggesting that zebra mussels could be an effective model for screening potential causative factors of MIH in humans. BPA and cadmium were toxic to zebra mussels and interfered quantitively with shell biomineralization. However, further studies on microstructure alterations and the exact interaction mechanisms are necessary to deepen the understanding of the mussel biomineralization model.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

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4. Paper II

Stefanie Kamann, Eva Oppel, **Fangfang Liu**, Franz-Xaver Reichl, Lutz Heinemann, and Christof Högg. Evaluation of isobornyl acrylate content in medical devices for diabetes treatment. *Diabetes Technology & Therapeutics*, 21(10), 533-537, 2019.

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ORIGINAL ARTICLE

Evaluation of Isobornyl Acrylate Content in Medical Devices for Diabetes Treatment

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Abstract

Background: Along with increased usage of continuous glucose monitors, flash glucose monitors, and patch pumps by patients with diabetes, the frequency of skin reactions has also increased. Skin irritation and itching can be annoying to users. However, more serious contact allergies to one or more components of the adhesives or plastic material of the housing of the devices can become lifelong. Redness and itchiness are so strong that patients can no longer use a particular system. In August 2017, a major culprit allergen, isobornyl acrylate (IBOA), was identified for these more serious reactions.

Objectives: Our objective was to evaluate IBOA content in different medical products.

Methods: The plastic material used for the housing of the Freestyle Libre ($n = 3$), Dexcom G6 ($n = 3$), and Enlite ($n = 4$) was analyzed for IBOA content by gas chromatography–mass spectrometry. Adhesives of the different systems were also analyzed.

Results: IBOA was found in the housings of Freestyle Libre and Enlite sensor, but not in the Dexcom G6.

Conclusions: Patients with an IBOA allergy should consider switching to a medical product without IBOA. Furthermore, removal of IBOA from devices that contact the skin is encouraged.

Keywords: Acrylates, Adhesives, Allergic contact dermatitis, Contact allergy, Continuous glucose monitoring, Flash glucose monitoring, Isobornyl acrylate, Patch test.

Introduction

SKIN REACTIONS TO externally applied diabetes products have become more common with the increased usage of these systems for treatment of patients with diabetes. Some months after market launch of the Freestyle Libre glucose monitoring system (Abbott, Chicago, IL), skin reactions with this system were described.[1] Several subsequent reports described skin reactions with this system[2–5]; however, in most of these studies, it was not differentiated between irritative and contact allergic skin lesions.[4,5] In adults, such device-related skin reactions are even associated with an increased patient scoring on the generic questionnaire Problem Areas in Diabetes on having problem areas in diabetes.[6] Although

more common, skin irritations do not occur with every wear or with the same intensity. Patients notice redness and sometimes itching. Irritation is usually due to individual and physical factors.[7] Individual factors may be the age or a predisposition to dry skin. Physical factors are due to the long skin occlusion under a sensor, sweating, or damaging the epidermis by rapidly tearing off the adhesive.

Allergic contact dermatitis (ACD) is less common, but of a greater clinical significance. ACD may be due to substances contained in the adhesives of the medical products or the plastic materials that are used for housing of the technical components themselves. This means that one or more allergens that are in the given medical product itself and/or the adhesive leads to an allergic type 4 contact allergy reaction.[8]

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It can take several weeks or even months until such a contact allergy develops; however, once the patient is sensitized to an allergen, this reaction remains likely lifelong and appears with every contact within a short period of time after another exposure.[9] Allergic skin reactions are mostly of stronger intensity than skin irritations. In case of Freestyle Libre, it occurs under the system and is so pronounced, that the system has to be removed after only a few hours or days. Redness and blistering appear, often with yellowish exudate (Fig. 1). In addition to the long exposure time and skin barrier disorders, predisposition factors for the development of this contact allergy relate to the amount and allergenic potency of one or more allergens. The patch test is according to the American Academy of Allergy, Asthma, and Immunology, ''the gold standard for contact allergen identification.''[9]

Reports of allergic skin reactions to medical devices for the treatment of diabetes were unusual before 2017. In 1995, two patients with diabetes were described that showed allergic reactions to the adhesives of the infusion set of their insulin pump.[10] Isobornyl acrylate (IBOA) was identified to be one of the components contained in the UV-cured glue used to fix the infusion set needle to its plastic stand. In 2016, the case of a child was published developing ACD toward a Dexcom G4 CGM.[11] A skin sensitivity patch test revealed a positive reaction to hydroxycyanoacrylate, which was present in the intermediate layer of the kit. Two other adults in whom the ACD was caused by 2-ethyl cyanoacrylate contained in their

FIG. 1. Allergic skin reaction of a boy after usage of Freestyle first generation.

Dexcom G4 were also described.[12] The manufacturer eliminated exposure that might trigger a skin reaction from the intermediate adhesive layer by attaching the CGM system to the adhesive using a thermic ultrasound technique.[13]

The major allergen generating reactions to the Freestyle Libre system was unknown until the summer of 2017 when a group of Belgian and Swedish dermatologists showed that in 12 of 13 of their affected patients with ACD, this was induced by IBOA.[3] In a subsequent study, we tested IBOA 0.1% with a boy with a known ACD induced by wearing the Freestyle Libre. There was a positive skin reaction after 48 and 72 h in the patch test.[4] However, the two original adhesives ([1] skin adhesive and [2] thin layer glue that sticks the sensor to the skin adhesive) that were provided by the manufacturer of these (Adhesive Research, Glen Rock, PA) did not show a positive skin reaction. Thus, our assumption was that IBOA may originate from the plastic material of the Freestyle Libre itself and subsequently diffuse through the plaster to the skin; at least in some patients the outline of the skin reactions is that of the housing of the Freestyle Libre and not that of the adhesive. IBOA could also be detected by gas chromatography (GC) in the housing of the patch pump Omnipod, but—like the Freestyle Libre—not in the adhesive that sticks the pump to the skin.[14,15] In contrast, it was reported that the housing of the Dexcom G5 and its adhesive are free from IBOA and are well tolerated by patients who are allergic to Freestyle Libre and IBOA.[16]

At present, no good estimations are available about how many patients suffer from ACD after IBOA sensibilization. This is, in part, due to the fact that ACD occurs typically after months of usage of the Freestyle Libre, even years later. In most clinical studies - which have a too short study duration - it was not differentiated between skin reactions that were simple irritations and real ACD.[5,6] A systematic evaluation of skin reactions, especially ACD, is missing.

The aim of our study was to estimate the IBOA content of a number of medical products that are applied to the skin for diabetes therapy.

Material and Methods

The IBOA content of different CGM systems (Freestyle Libre [Abbott]; G6 [LOT 5249363; Dexcom, Inc., San Diego, CA]; Enlite [sensor LOT D138P, transmitter LOT GT6066295M; Medtronic, Dublin, Ireland]) was measured.

Sample preparation

The adhesives of the CGM systems were manually removed from the housings. The CGM systems (Freestyle Libre ($n = 3$), Dexcom G6 ($n = 3$), and Enlite sensor ($n = 4$) were transferred into weighing bottles (45 mL, 60×30 mm; neo-Lab, Heidelberg, Germany). Six milliliter of methanol (GC Ultra Grade, RATISOLV® ≥99.9%, Roth, Karlsruhe, Germany) was added to immerse the bottom side (dermal contact side) of the systems in a solvent level of ~2 mm. This corresponds to immersed areas of ~9.0 cm² (Freestyle Libre), 7.0 cm² (Dexcom G6), and 2.8 cm² (Enlite). As internal standard caffeine (CF) solution (0.01 mg/mL) (HPLC ≥99.0%, Sigma-Aldrich, St. Louis, United States) was added. After 3 days incubation of the systems in the dark at room temperature, the IBOA content in the methanol (eluates) were
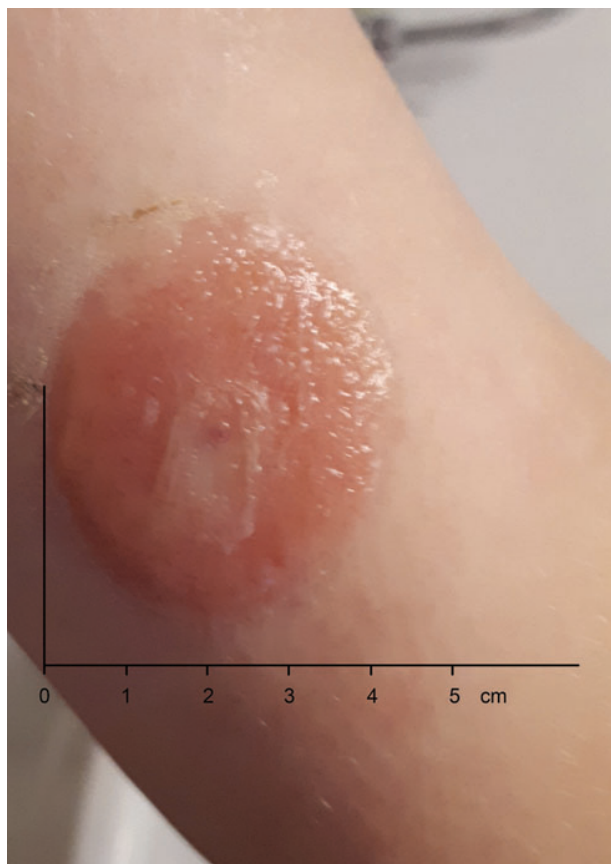
analyzed (see Analytic procedure) by GC–mass spectrometry (GC/MS).

Water (LC-MS-Grade, ROTISOLV®; Roth) elutions were carried out as described previously. For GC/MS analysis, water samples previously were extracted one time with ethyl acetate (LC-MS-Grade, ROTISOLV ≥99.9%) (1:1 v/v). To optimize layer separation, the samples were centrifuged at 2800 rpm for 10 min.[17]

The same technical procedure was conducted on pieces of the adhesives of the CGM systems (the adhesive of the Freestyle Libre was supplied by Adhesive Research; skin plaster $n = 3$ and transparent glue layer that fixes the sensor to the skin adhesive $n = 3$; Dexcom G6 ($n = 3$) and Enlite ($n = 1$).

Analytic procedure

The analysis of the eluates was performed on a Finnigan Trace GC ultra gas chromatograph connected to a DSQ mass spectrometer (Thermo Electron, Dreieich, Germany). A J&W VF-5ms capillary column (length 30 m, inner diameter 0.25 mm; coating 0.25 μm; Agilent, Böblingen, Germany) was used as the capillary column for gas chromatographic separation. Helium 5.0 was used as carrier gas at a constant flow rate of 1 mL/min. The temperature of the transfer line was 250°C. For sample analysis, 1 μL each was injected in splitless mode (splitless time 1 min, split flow 50 mL/min). For capillary transfer, the programmable temperature vaporizing inlet was heated from 30°C to 320°C (14.5°C/s) and finally held for 5 min at this temperature. The GC oven was initially heated isothermally at 50°C for 2 min, then increased

to 280°C (25°C/min), and finally remained for 5 min at this temperature. The MS was operated in the electron impact mode at 70 eV (ion source temperature: 240°C). Samples were recorded in full scan mode (m/z 50–600).

Identification of IBOA was achieved by comparing the mass spectra and retention time with those of the reference standard IBOA (Merck, Darmstadt, Germany). For the reference standard IBOA, a calibration was performed. Resulting limit of quantification (LOQ) for IBOA in water (ethyl acetate extract) was 0.20 μg/mL and LOQ for IBOA in methanol was 0.10 μg/mL. The quantity of identified IBOA was calculated by correlating its characteristic mass peak (m/z 55) area to the corresponding predetermined calibration curve (internal standard CF). An example chromatogram of Freestyle Libre is shown in Figure 2.

Results

In the water eluates of all CGM systems—including the adhesives—no IBOA (<LOQ) could be detected; however, at least in some of the methanol eluates of the CGM systems, it could be detected (Table 1). In case of the Freestyle Libre, 2.64 μg/mL with a standard deviation (SD) of 0.36 μg/mL of IBOA (3 days, methanol) corresponds to a dose/area of ~ 0.29 μg/cm^2 per mL (immersed surface area). In both types of adhesives of the Freestyle Libre, no IBOA (<LOQ) was found. In the methanol eluates of Dexcom G6 sensors and its adhesives, no IBOA was detected. With the Enlite CGM system, IBOA was found both in the methanol eluates of the sensor (1.11 μg/mL with a SD of 0.12 μg/mL corresponds to a
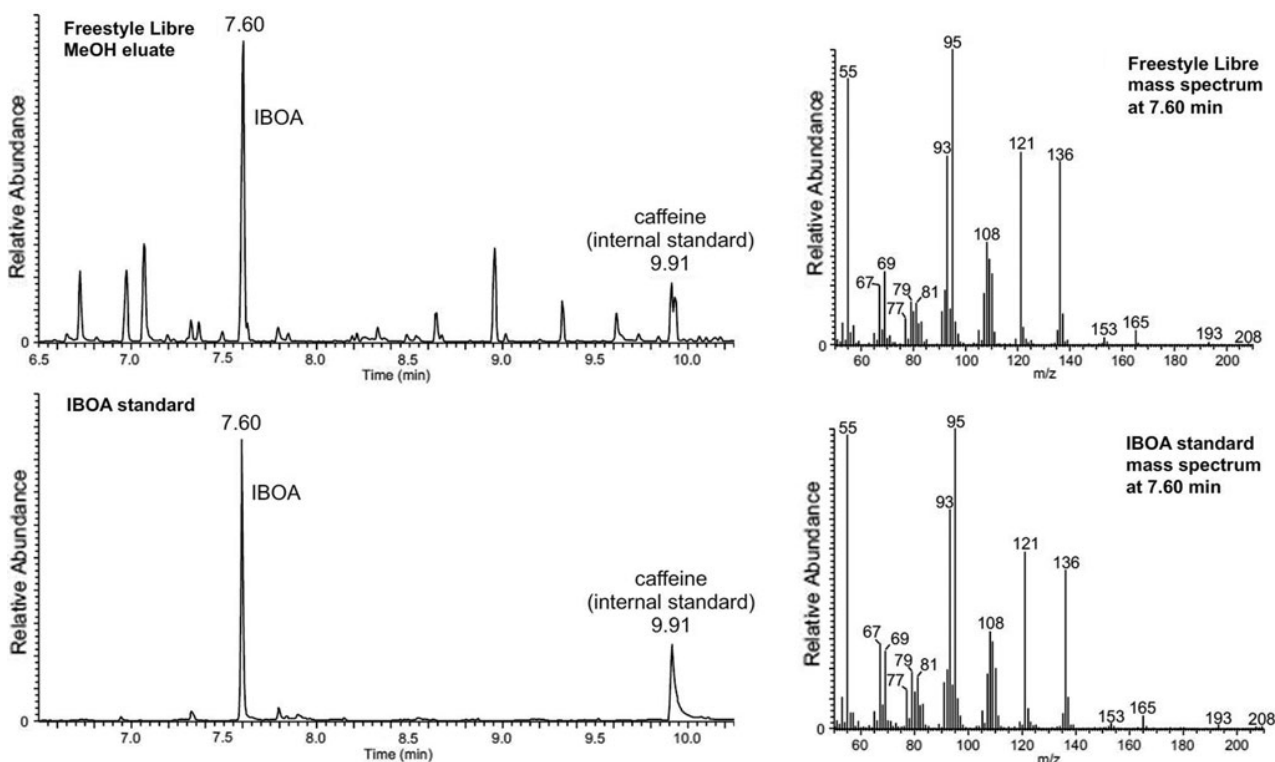
FIG. 2. Example chromatograms of Freestyle Libre methanol eluate (above) in compression with IBOA reference standard (below). Retention time of IBOA in standard (below) and sample (above) was 7.60 min. Corresponding mass spectra of sample and standard yielded identical patterns.

TABLE 1. THE ISOBORNYL ACRYLATE CONTENT
IN THE HOUSING AND ADHESIVES OF DIFFERENT
MEDICAL DEVICES USED FOR CONTINUOUS GLUCOSE
MONITORING IN PATIENTS WITH DIABETES
WAS ANALYZED BY GC/MASS SPECTROMETER AFTER 3
DAYS ELUTION IN METHANOL AND WATER (CONTROL)

Medical device	n	Methanol 3 days, mean ± standard deviation (µg/mL)	Water 3 days (µg/mL)
FL sensor	3	2.64 ± 0.36	<LOQ
FL skin adhesive	3	<LOQ	<LOQ
FL thin layer glue	3	<LOQ	<LOQ
Dexcom G6 CGM system	3	<LOQ	<LOQ
Dexcom adhesive	3	<LOQ	<LOQ
Enlite CGM system	4	1.11 ± 0.12	<LOQ
Enlite transmitter	1	<LOQ	<LOQ
Enlite adhesive	1	0.26	<LOQ

CGM, continuous glucose monitoring; FL, Freestyle Libre; LOQ, limit of quantification.

dose/area of ∼0.40 µg/cm^2 per mL [immersed surface area]) and the adhesive (0.26 µg/mL), but not in the eluates of the transmitter.

Discussion

IBOA is an acrylate used widely in the automobile industry for UV and weather protection.[18] In safety data sheets, it is classified as an irritant for skin and eyes. In August 2013, Christoffers et al. carried out a study with various acrylates and concluded that IBOA does not have to be included in the skin patch test.[19] However, data obtained in this study revealed that IBOA is detectable in the housing of the Freestyle Libre and the Enlite sensor and the adhesive of Enlite. This suggests that an ever increasing number of patients with diabetes are being exposed to IBOA for longer periods of time. We recommend that IBOA be included in skin patch tests for evaluating the sensitivity of a given patient to this substance. In this context, it is of interest to know that IBOA 0.1% will become available as a commercial test substance sometime in the middle of 2019.

This study confirms the finding of IBOA in the housing of the Freestyle Libre, and also finds that smaller amounts of IBOA can be detected in the Enlite CGM system and in its corresponding adhesive. In the Dexcom G6 and its plaster, no IBOA could be measured; a finding that confirms former measurements of the G5.[16] A limitation of our study is that the measurements were not performed in a "blinded manner." Our measurement results should be confirmed by measurements of samples from a larger number of devices.

Once a patient is sensitized to an allergen, future contacts with this substance triggers a reaction again, commonly known as a memory response. So it is most likely that patients will have it for the rest of their life.[9]

So, once a patient is allergic to IBOA, this contact allergy lasts surely for the rest of his or her life and he or she also may be at risk to develop cross-allergic reactions to other acrylates. This should be avoided at all costs. Cross-reactions mean that patients can also react toward other materials and adhesives that contain structures that are chemically similar, which might be used for other medical reasons to such pa-

tients. IBOA has a very special branched structure and fortunately may be not so much at risk to develop cross allergies; however, it has a high allergenic potency itself.

In case of the Freestyle Libre, a relevant amount of IBOA seems to be present. In view of the large number of users of this CGM system (>1.5 million on a worldwide basis), it is hoped that the manufacturer comes along with an IBOA-free version soon. IBOA appears to be the major allergen with this CGM system. Recently, another relevant allergen was identified in the housing of the Freestyle Libre, called N,N-dimethylacrylamide.[20] It is worth considering as well the acrylates of the adhesive or other acrylates contained in the housing. Some patients appear to develop ACD toward these substances.

Patients with an IBOA allergy should avoid contact to this allergen; however, most patients do not want to give up their CGM system due to the benefits they have by using it;

FIG. 3. Addition of hydrocolloid-based adhesives/plates in a patient with an IBOA allergy, preventing allergens from migrating into the skin from the sensor set and thus decreasing allergic contact dermatitis symptoms. IBOA, isobornyl acrylate.

therefore, they do everything to be able to continue using, for example, the Freestyle Libre. Patients first tried to fix special skin plates under their sensors, yet most constructions did not appear to work over a longer period, or glucose concentrations were not accurately being measured in the same skin compartment due to a change in the depth of the needle tip of the sensor. Good experience was reported with some hydrocolloid-based thicker adhesives/plates, for example, blister plasters.[21] Such constructions do work not only in patients with an IBOA allergy but also in patients who have other severe skin reactions (Fig. 3).

In conclusion, the acrylate IBOA was detectable in the housings/adhesives of some CGM systems and a patch pump (X), but not in all. In view of the severity of allergic skin reactions and the potential clinical consequences, the manufacturer should develop IBOA-free housings and adhesives.

Author Disclosure Statement

No competing financial interests exist.

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