



Defining corneal chemical burns: A novel exact and adjustable ocular model

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ABSTRACT

Introduction: Live-animal-free ocular toxicity models and tests are a necessity in multiple branches of medicine, industry and science. Corneal models with adjustable ranges of injury severities do not exist. In this work, a novel and precise and dose - response method to induce and observe *ex vivo* corneal chemical burns has been established.

Methods: The EVEIT (Ex Vivo Eye Irritation Test) is based on an *ex vivo* corneal organ model for rabbit corneas from food industry. Further, a highly precise three - axis workstation has been employed to apply liquid corrosive, sodium hydroxide (NaOH), droplets in a nanolitre (nL) range onto the corneal surface. Optical Coherence Tomography (OCT) has been used to observe and quantify the elicited changes in the corneal layers.

Results: The speed and intervals of single nanodroplet application played a crucial role in the extent of the corneal changes. Similar total volumes applied at low frequencies elicited deep and extensive changes in the corneal layers whereas high application frequencies elicited comparatively superficial changes. Increasing NaOH concentrations effected measurably increasing corneal changes. Increasing the volume of applied NaOH also showed an increase in corneal changes.

Conclusions: OCT imaging proved to be effective in observing, documenting and quantifying the changes in the corneal layers. The *ex vivo* model, in conjunction with the novel application method was able to induce and display distinctive and consistent correlations between NaOH volume, concentration and elicited corneal changes. This *ex vivo* ocular chemical burn model provides a consistent *in vitro* basis for pharmaceutical and toxicological experiments and investigations into corneal chemical burn mechanisms and treatment.

1. Introduction

The necessity for live animal free corneal toxicity tests is a political and social demand. The need for reproducible and defined test strategies is of increasing interest in various branches of medicine, industry and science. The Ex Vivo Eye Irritation Test (EVEIT) provides a platform for such a test. Our aim is to have a precise definition of area and depth [1] derived from known corrosives to detect the qualities of unknown substances with the same application pattern, thus providing a highly reproducible application and exposition of chemicals towards the cornea. The EVEIT employs an *ex vivo* organ culture which utilizes rabbit corneas from food industry. While the cultured cornea is being supplied with nutrients from the endothelial side, physiological flow and pressure conditions are granted by the artificial anterior chamber. The EVEIT system provides epithelial healing after mechanical or chemical damage

as unique specification [2]. In this work, we introduce a novel automatic contact-free substance application method, which induces area, depth and time defined chemical corneal burns. In previous works by other groups it has been shown, that the extent of cell death in a chemical ocular burn scenario correlates with the area and depth of the corneal chemical injury [3]. Subsequently, the detected area and depth of the changes in the corneal layers are a probable way to define and describe the extent and severity of injury. Further investigations connected these data to the predicted injury development over time [4]. It has been repeatedly proposed, that the extent of the initial injury could be used as predictive model for the overall irritancy of a substance [1,5,6]. In these investigations, the group used confocal microscopy and histology as detection methods. Further, it has been proposed that this could be used as a quantitative approach as compared to the suggestively subjective evaluation methods of the *in vivo* Draize Test. As a basis for the

Abbreviation: EVEIT, Ex Vivo Eye Irritation Test.

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development and validation of alternative methods to the *in vivo* test method this would then serve as a consistent dataset.

In this study we used Optical Coherence Tomography (OCT) to acquire direct *ex vivo* optical cross sections of treated EVEIT corneas. For this, a novel automated and highly precise application mechanism was employed to place sodium hydroxide droplets in a nanolitre (nL) range onto the corneal surface. Automated digital image analysis software was used to quantify the measured cross-sectional area and depth of the detected changes in the cornea. In addition to this, conventional macroscopic evaluation with live fluorescein staining was done. We find, that with our novel application method we are able to place test substances onto the corneal surface with clearly measurable distinctiveness and are able to describe the ensuing corneal changes based on OCT data.

2. Materials and methods

2.1. Corneal organ culture

The Ex Vivo Eye Irritation Test (EVEIT) is based on an *ex vivo* rabbit corneal model [7]. The primary tissues are acquired from food industry. Briefly, the corneal tissues (including the limbus region) are isolated and placed into a specialised cultivation chamber. In this cultivation chamber (EVEIT-System), the rabbit cornea is supplied with nutrients (MEM with stable glutamine; supplemented with 2.2 g/L NaHCO₃, 5.8 g/L HEPES, amikacin 0.2 g/L, piperacillin 2 g/L, and amphotericin B 2.5 mg/L; adjusted to pH 7.4 with 0.25 M NaOH) from the endothelial side, whereas the epithelial side is exposed to the conditions inside the incubator (32 °C; 99 % humidity). Via the artificial anterior chamber physiological flow and pressure conditions (15 mmHg; 6.44 µL/min) are being approximated.

2.2. BioSpotter

The automated substance applicator (BioSpotter, BioFluidix-Freiburg Germany; Fig. 1) provides a platform for the automated – high throughput Ex Vivo Eye Irritation Test (EVEIT) [7]. The BioSpotter is a robot with a three – axis – actuator system being designed for automatic dosing multiwell plates. We used a modified version that has been optimized to apply test substances onto the curved surface of our cultivated *ex vivo* corneas. The system is equipped with a patented fluid

application mechanism. Up to 8 fluids (corrosives, therapeutics) can be applied in exactly repeated localized spots with defined volumes onto a single cornea within seconds. Due to comparability and biological issues of non-interference of single spots, we decided to use circular patterns around the corneal centre with a defined distance to corneal centre and limbus. These substance patterns are repeated on up to 6 corneas in one work cycle. The integrated camera automatically documents macroscopical corneal changes.

The dispensing mechanism is based on the ability of the system to apply an exact, very short (millisecond range) kinetic impulse to a substance filled capillary disrupting and expelling a microdroplet from the capillary tip. This is realized by employing a piezo based impulse mechanism that translates kinetic force to the side of a disposable, polymeric capillary named “pipe” in the further text. The following, very short pressure changes inside the pipe result in the formation of a consistent and quantifiable droplet that is accelerated toward the corneal surface. The calibration of the droplet volume is done via stroboscopic high speed camera images and volumetric adaptation of repeated droplets applied at a separate calibration spot. The documentation of morphological changes of the corneas is done by a high resolution digital camera looking onto the corneas from upside down. A ring of white light LEDs and a high power single wavelength blue LED were installed to provide illumination and excitation for sodium-fluorescein surface staining dye. An automated rinsing of corneal surfaces with defined solutions and a simultaneous aspiration of excess fluid is integrated in the machine.

2.3. Application protocol

After 24 h the EVEIT corneas are examined for epithelial integrity, biochemical stability and clearness. After these corneas passed the quality control, an initial humidification of the corneal surface was conducted with a specialised sponge (Sugi® Eyespear blunt tip, Kettenbach, Germany) which was soaked in Ringer solution (B. Braun, Germany). Following this, the corneal surfaces were treated with different concentrations and volumes of sodium hydroxide. The time between initial humidification and application was exactly quantified to discover possible relations between surface humidification status and penetrative effect of the corrosive substance. The exposure was performed in portions of single 10 nL droplets. A range of 10 nL up to 80 nL of test substance was used. The incremental volumes were repeatedly



Fig. 1. BioSpotter (BioFluidix, Freiburg, Germany): 3 axis of application are indicated as directions (green arrows); the high resolution camera for documentation, cornea recognition and mapping (blue arrow); stroboscopic camera for volume calibration (red arrow); working surface (white arrow); integrated computer system for control (yellow arrow); dispensing mechanism (orange arrow). (For interpretation of the references to colour in this Figure legend, the reader is referred to the web version of this article).

applied under a defined frequency to one single position onto the cornea until the targeted total volume was reached. Depending on the experimental design this repeating frequency ranged from more than 10 droplets per second (10 Hz) down to 5 droplets during 10 s (0.2 Hz). After the local application a 30 s incubation period was followed by an automated 20 s rinsing with Ringer solution. Immediately after rinsing OCT images were recorded. Macroscopic photos were taken after all positions were treated.

2.4. Optical coherence tomography (OCT)

Optical cross-sections of the treated corneal surfaces were measured immediately after the exposure procedure. A Ganymede Spectral Radar (Thorlabs, Germany) was used according to previous work [7,8]. The cultured and treated EVEIT- corneas were adjusted for measurement. The measurements were made across a 4 mm range of width with a resolution of 270 columns with an averaging level of ten resulting in a lateral resolution of 14 μm . The depth resolution is around 2 μm . To measure the full extent of the corneal changes, the OCT cross-section was adjusted to reach maximum signal. The raw data were exported and converted in scale to a .jpeg file for further quantification.

2.5. Quantification of OCT signals

The signal quantification was performed on the transformed measurements. The free software ImageJ (Fiji, RRID:SCR_002285) was employed to perform the image analysis. For this, the exported OCT image was transformed with the “RGB to Luminance” - function into an 8-bit greyscale image. A binary transformation was performed on this image with the “Make Binary” -function. Finally, the binary image was analysed with the “particle analysis” function (size 0 – infinity; circularity 0–1) to quantify the signal area of the cross-section (Fig. 2). Furthermore, the corneal thickness at the site of the injury was measured together with the depth of the corneal signal change to obtain relative values.

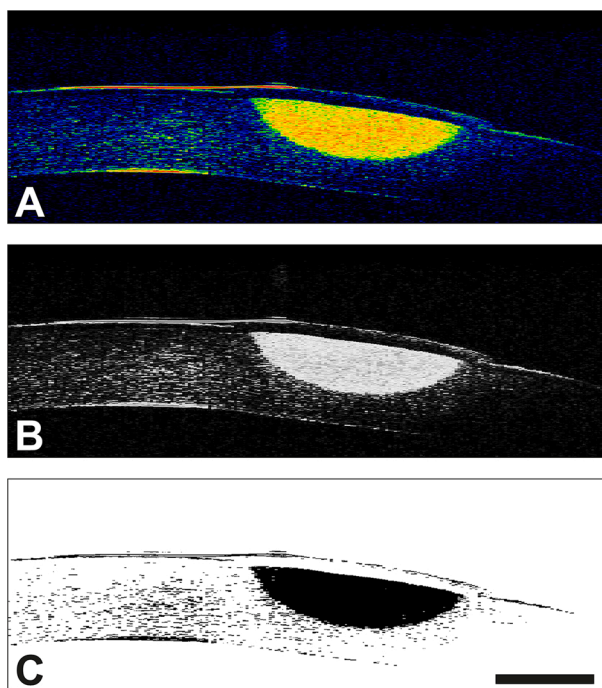


Fig. 2. Quantification of OCT measurements: The exported data (A) were transformed via the RGB-to-Luminance option (B) and subsequently transformed via the Make-Binary option (C). The Analyse-Particle option detected and quantified the signal. Scale bar: 500 μm .

2.6. Fluorescein staining and macroscopic assessment

Macroscopic assessment under white light and fluorescein staining was performed as described before [7–10]. All corneas were macroscopically evaluated initially under white light. Then a 0.17 % (w/v) of sodium fluorescein in isotonic Ringer solution was applied onto the corneal surface. After rinsing of excess dye the cornea was imaged under a cobalt glass filtered blue light. All images were analysed by measuring the fluorescein positive surfaces via ImageJ (Fiji, RRID:SCR_002285).

2.7. Statistics

Data was processed with statistics software Prism® (GraphPad Prism, RRID:SCR_002798). Three - parametric, non - linear curve fittings were conducted on the various datasets and the R-Square value was generated. The regression was constrained to zero as bottom limit. In addition to this, Student’s t - test (paired, two - tailed) was conducted to detect significant differences between the individual curves in their respective datasets. P values below or equal to 0.05 were considered as statistically significant. The Person coefficient was generated to show possible correlations between the applied total test substance volume and the respective signal extent.

3. Results

3.1. Incubation time between initial humidification and exposure has minimal effect

In a preliminary trial, the corneal positions were treated with increasing 10 nL droplet numbers of 1 M NaOH at a more than 10 Hz droplet frequency. We found that the status of humidification on the cornea was a crucial factor to promote or inhibit diffusion. This led to exactly defining the delays between corneal pre - humidification and application of the corrosive substance onto the corneal surface. EVEIT corneas have been treated with increasing incubation times between initial surface humidification and substance application (Fig. 3). The humidification was performed with a Ringers solution pre-soaked eye sponge. This soft wetted sponge was moved gently over the whole corneal surface to achieve a stable and total surface wetting. As observed in previous experiments the penetration depth of NaOH into corneal layers ranged from superficial to extensive (data not shown).

The penetrative effect which was observed in this trial was much less extensive as expected (Fig. 3 C). Weak correlations between the cross-sectional OCT signal areas and the applied test substance volumes were detected (Fig. 3 E; Table 1). It was observed that the OCT signals have the tendency to be slightly more extensive when the incubation time after the initial humidification was increased (Fig. 3 C). Conversely, the correlations and curve fittings of the fluorescein positive surface area to the applied test substance volumes were more distinct (Fig. 3 B; Table 2). Few notable differences between incubation times were observed with the fitted curves of the fluorescein signal quantifications (Fig. 3 B, Table 2).

3.2. Changes in application frequency have a clear impact on penetrative effect

In another experiment the impact of the droplet frequency on the extent of corneal injury has been investigated (Fig. 4). As of yet observed penetration depths elicited by application via BioSpotter with high frequency (10 Hz) were mostly superficial (Fig. 3 C) while manual application methods done before [7] resulted in a more extensive penetration. In these previous works a Perspex cylinder filled with the test substance was placed onto the corneal surface which made the upholding of the local test substance concentration and the reduction of lateral surface diffusion possible. To compensate for this in this study, repeated spots were applied to the same position on the cornea to uphold

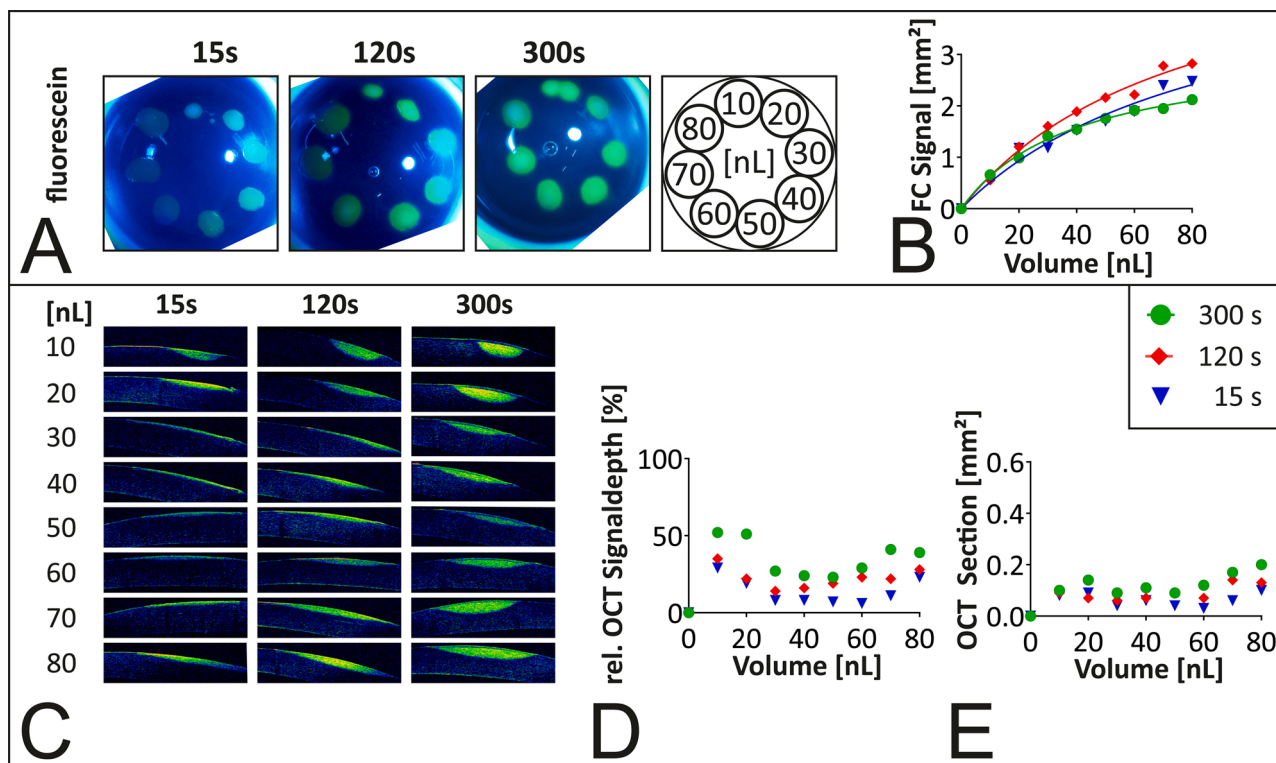


Fig. 3. Effect of differing incubation times between initial surface humidification and substance application (15 s, 120 s and 300 s). The *ex vivo* rabbit corneas were sequentially treated with 1 M NaOH. The test substance droplet count was increased in 10 nL increments to reach the respective total volume. Macroscopic assessment (A) – and quantification (B) of the fluorescein positive corneal surface changes and OCT – measurements of corneal depth profiles (C). The green-yellow signals signify optical changes in the corneal layers caused by the treatment. Relative depth (D) - and area (E) – quantification of OCT cross sections. (For interpretation of the references to colour in this Figure legend, the reader is referred to the web version of this article).

Table 1

Curve fitting and comparison of cross sectional OCT measurements (Fig. 3); Non-linear curve fitting; correlation of OCT signal extent with the used test substance volume; t-test of the respective graph data sets (paired, two tailed).

	Non-linear Curve Fit (R square)	Correlation Signal to Volume (Pearson r)	T - Test (p - value)	15 s	120s	300s
15 s	0.4328	0.3171	–	–	0.02705	0.00049
120 s	0.5939	0.7807	0.02705	–	–	0.00229
300 s	0.647	0.7827	0.00049	0.00229	–	–

Table 2

Curve fittings and comparison of macroscopic, fluorescein positive surface signals (Fig. 3); Non-linear curve fitting; correlation of fluorescein surface signal extent with the used test substance volume; t-test of the respective graph data sets (paired, two tailed).

	Non-linear Curve Fit (R square)	Correlation Signal to Volume (Pearson r)	T - Test (p - value)	15 s	120s	300s
15 s	0.9711	0.9779	–	–	0.0048	0.3939
120 s	0.9898	0.9755	0.0048	–	–	0.0126
300 s	0.9966	0.9505	0.3939	0.0126	–	–

the local test substance concentration. 15 s after initial humidification of the cornea 1 M NaOH was sputtered onto the cornea in 10 nL increments.

The macroscopic evaluation of the tested corneas showed few notable differences between the different application modes (data not

shown). A drastic increase in OCT signal - intensity, - depth and area was noted with the change of the spotting mode (Fig. 4 A): The mode which delivered the test substance droplets with a high frequency yielded largely superficial OCT signals (Fig. 4 A). Conversely, the modes in which 1 or 0.2 Hz were used, showed a notably more extensive OCT depth signals. Here, it was noted that 1 s or 5 s idle time between spots have a comparable effect (Fig. 4, Table 3).

3.3. Increasing test substance concentrations elicit discreetly proportional effects

After investigating the penetrative behaviour of the test substance in different modes of application, the test substance was characterised in different concentrations to observe the precision and detection range of the method. For this, four different concentrations of NaOH (260 mM, 500 mM, 1000 mM, 2000 mM) were employed. One EVEIT cornea for each concentration was used. On each cornea eight different volumes (10, 20, 30, 40, 50, 60, 70, 80 nL) have been applied in a circular pattern around the corneal apex. 15 s after the initial humidification with a sponge as previously described, NaOH was applied by the BioSpotter to the corneal surface in 10 nL increments. The application frequency of 0.2 Hz was as used. After a 30 s incubation time, the cornea was rinsed with Ringer solution for 20 s. Photographic fluorescein and OCT measurement were done as described before.

The macroscopic assessment of the processed corneas showed a consistent increase in opacity, seen as surface signal, the higher the NaOH concentration was. Furthermore, opacity increased in the cornea by increasing of the used substance volume (data not shown). A more pronounced difference between applied concentrations was observable with OCT (Fig. 5). The cross sectional OCT measurements of the treated corneas showed an increased extent of area - and depth signals correlating with the increase of substance concentration (Fig. 5).

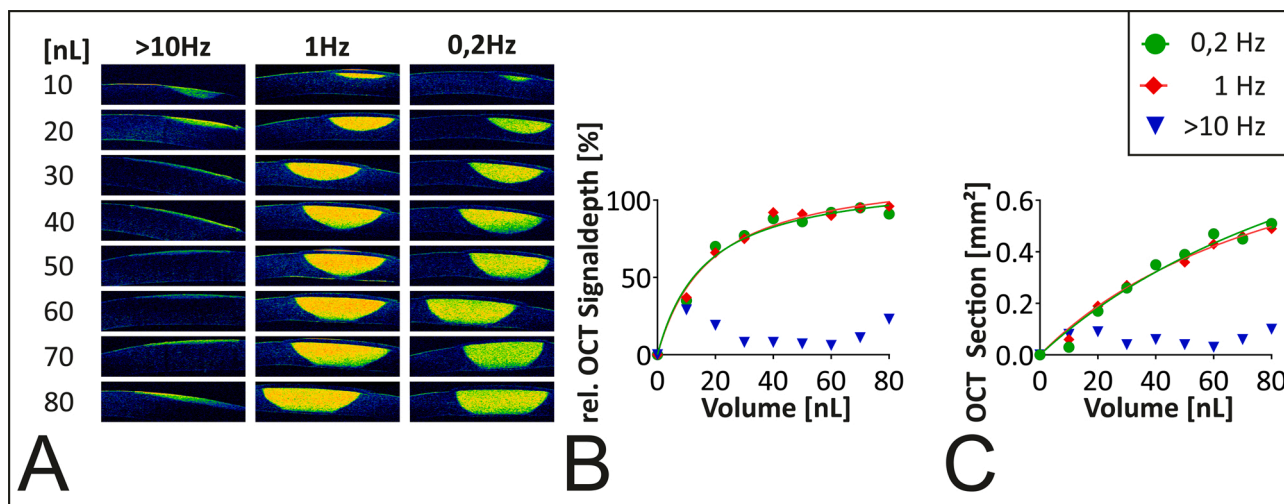


Fig. 4. OCT-measurements of corneal depth profiles with varying application frequencies (> 10 Hz; 1 Hz; 0.2 Hz). The *ex vivo* rabbit corneas were sequentially treated with 1 M NaOH. The test substance droplet count was increased in 10 nL increments to reach the respective total volume. The green-yellow signals signify optical changes in the corneal layers caused by the treatment (A). The OCT data from the >10 Hz sample has also been used in Fig. 3. Relative depth (B) - and area (C) – quantification of OCT cross sections. (For interpretation of the references to colour in this Figure legend, the reader is referred to the web version of this article).

Table 3

Curve fitting and comparison of cross sectional OCT measurements (Fig. 4); Non-linear curve fitting; correlation of OCT signal extent with the used test substance volume; *t*-test of the respective graph data sets (paired, two tailed).

	Non-linear Curve Fit (R square)	Correlation Signal to Volume (Pearson r)	T - Test (p - value)	>10 Hz	1 Hz	0.2 Hz
>10 Hz	0.4328	0.3171	–	–	0.00172	0.00321
1 Hz	0.9873	0.9741	0.00172	–	–	0.81355
0.2 Hz	0.972	0.9712	0.00321	0.81355	–	–

Additionally, the signal extent increased within their respective corneas the higher the used substance volume was (Fig. 5, Table 4). In higher concentrations of NaOH there was no further difference in macroscopical analysis (data not shown) of the surface but a significant difference under the surface (Fig. 5 A; Table 4). The extent and depth of the corneal penetration intensified with the increase of the used substance

concentration. Conversely, similar to their macroscopic evaluations, the corneas that have been treated with 1000 mM and 2000 mM NaOH have comparable penetration depths (Fig. 5 B). However, the extent of the lateral penetration was notably bigger with the 2000 mM treatment (Fig. 5 C).

4. Discussion

In this study, we aimed to establish a new application system in our EVEIT protocol that produces automatic repetitive and highly repeatable ocular chemical damage with varying degrees of extent. We were able to elicit consistent chemical burns that ranged from slight to extensive with corneal permeation ranging from superficial to full thickness. To our knowledge, an *ex vivo* based ocular burn model with finely adjustable injury severity has, as of yet, not been reported.

4.1. The application frequency of the test substance droplets influences the corrosive corneal penetration intensity

As shown in this work, when the droplet frequency was lowered, the

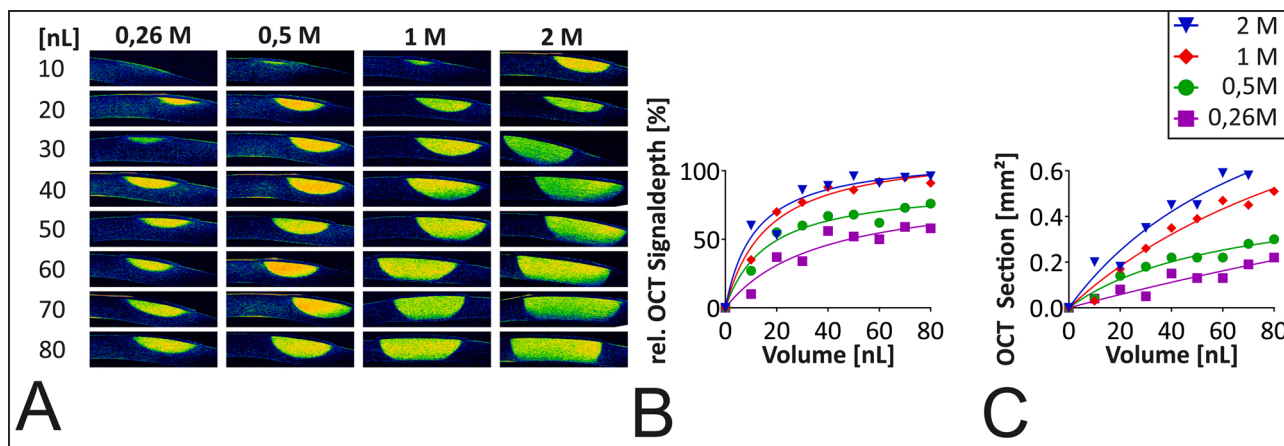


Fig. 5. OCT-measurements of corneal depth profiles with increasing substance concentrations (0.26 M; 0.5 M; 1 M; 2 M). The *ex vivo* rabbit corneas were treated with increasing concentrations of NaOH. The green-yellow signals signify optical changes in the corneal layers caused by the treatment (A). The test substance droplet count was increased in 10 nL increments to reach the respective total volume. Relative depth (B) - and area (C) – quantification of OCT cross sections. (For interpretation of the references to colour in this Figure legend, the reader is referred to the web version of this article).

Table 4

Curve fitting and comparison of cross sectional OCT measurements (Fig. 5); Non-linear curve fitting; correlation of OCT signal extent with the used test substance volume; t-test of the respective graph data sets (paired, two tailed).

	Non-linear Curve Fit (R square)	Correlation Signal to Volume (Pearson r)	T - Test (p - value)	260 mM	500 mM	1000 mM	2000 mM
260 mM	0.8933	0.9467		–	0.00052	0.00131	0.00022
500 mM	0.9635	0.9514		0.00052	–	0.00421	0.00055
1000 mM	0.972	0.9712		0.00131	0.00421	–	0.00078
2000 mM	0.9642	0.9667		0.00022	0.00055	0.00078	–

vertical burn direction notably intensified. This phenomenon could primarily be explained through the upkeep of the local surface - substance concentration gradient by the periodical renewal of the applied substance. The gradient-driven penetration / translation of the substance is therefore more effective than in the corneas where the volume is applied practically instantaneous with a high initial volume allowed to spread superficially. A secondary explanation for this difference in penetration depth can be that the substance is being laterally dispersed in the instantaneous (>10 Hz) application mode: The first droplet arrives on the corneal surface, but before the substance has time to interact with the surface and diffuse into the tissue, it is hit by the next droplet and dispersed to the sides. This theory is supported by the OCT measurements of the >10 Hz samples of corneas: In this more or less instantaneous application mode we found a larger penetration depth of the single 10 nL spot than the higher-volume spots. Thus, it seems, that an undisturbed droplet permeated the corneal layers relatively more than a chain of rapidly incoming droplets. In further proceedings the low-frequency-incremental application of test substances was preferred to optimize the effect.

4.2. A precise and controllable method for inducing and documenting corneal chemical burns

By increasing the NaOH test substance concentration we were able to show a very distinct change in the extent of the corneal injury severities. The surface damage is similar between concentrations because the substance comes, undiluted, in direct contact with the surface structures. Fluorescein does stain compromised epithelial surfaces and does not differentiate between varying penetration depths. Conversely, volume and concentration of the substance play a more substantial role when it comes to penetration depth and extent of lateral penetration into the corneal layers. The higher the applied volume was, the more corrosively active molecules were available to cause changes in the corneal layers. In addition to this, substance concentration and thus osmolality plays a crucial role in corneal penetration properties since the translation of the substance into the corneal layers is largely driven by diffusion physics.

4.3. A model for vertical ocular chemical burn behaviour

A reliable “dose – effect” relation has been discovered that was defined by two major parameters. Firstly, the surface must be prepared to be an acceptor, meaning that a specific humidity facilitates the surface interaction. Secondly, the application of corrosive substances needs time for diffusion. The time for diffusion of the corrosive into the tissue is needed to allow for surface cleavage of the applied substance before another droplet of corrosive is applied. If these conditions are kept, classical saturation kinetics can be achieved by increasing either the total mass of a corrosive substance or the concentration of the substance. The affected, cross-sectional area being mentioned by Maurer and Jester as “Area and Depth” [3,4,6] is correlated to the amount and concentration of the corrosive. We are confident that the usage of an OCT Imager is suitable for the determination and quantification of corneal depth changes in this context. These data confirm the clinical finding that the extent of eye burns are related to concentration of the corrosive

and the exposure time on the corneal surface [11]. In contrast to the Draize test, LVET in living animals and the BCOP or HET-CAM tests our approach aims to a reproducible delivery of corrosives and test substances for evaluation of the ocular toxicity onto a very small and exactly defined area of the cornea, whereas in all other before mentioned systems the substances are delivered by a higher volume onto the totality of the ocular surface. The former area and depth description of Maurer and Jester was the result of a whole conjunctival and corneal surface interaction with diffusion from a severely altered fluid film on the surface. Caustic substances caused severe alterations of the cornea everywhere where the epithelial surface did not withstand the chemical. In contrast to this we are now able to show a precise delivery of substances staying at the site and causing only local effect. Certainty of mass delivery is the first condition of evaluation of later effects being evaluated as possible toxicity.

Since we are fully aware that a partial *in vitro* model, as it is described here, will not accurately represent a complete animal context, it is not our aim to do so. The EVEIT itself is currently being validated as a specific test for ocular toxicity that can distinguish between reversible and irreversible corneal chemical injuries. However, in this study only the *ex vivo* cultivation method of this test is being utilized. Ultimately, the here employed method will be able to put the noxious properties of potentially harmful substances in relation to each other. To conclude, the ocular chemical burn model will be able to act as a consistent *ex vivo* basis for investigations into ocular injury severities and mechanisms.

Authors' contributions

MG created the data; MG, CP and NS planned the experiments reviewed, commented and interpreted the data; MG wrote the initial manuscript; CP and NS critically revised the manuscript.

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Ethics approval

No approval required. No live animal or human testing was involved in this work. No human tissues were involved no patient data were used. Animal tissues involved were procured as waste product from food industry which falls under the purview of the governmental German Veterinary office.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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