Die Anwendung des EVEIT-Tests in der Bewertung von Chemikalien und Medikamenten

Von der Medizinischen Fakultät der Rheinisch-Westfälischen Technischen Hochschule Aachen zur Erlangung des akademischen Grades einer Doktorin der Medizin genehmigte Dissertation

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Meinen Eltern

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List of abbreviations

AACM	Artificial anterior chamber medium
BCOP	Bovine Corneal Opacity/Permeability Test
C	Celsius (degrees)
CCL2	Chemokine (C-C motif) ligand 2
Cm	centimetres
CO	Carbon monoxide
CO_2	Carbon dioxide
CSIF	Cytokine Synthesis Inhibitory Factor
Dipl-Ing agr.	Diplomagraringenieur
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
e.g.	exempli gratia (for example)
etc.	et cetera
esp.	Especially
EVEIT	ex vivo eye irritation test
FGF	fibroblast growth factor
FGFR	fibroblast growth factor cell surface receptor
fig	figure
H_2O_2	hydrogene peroxide
HET CAM	Hen's Egg Test Chorioallantoic Membrane
i.e.	id est (that is)
IL	interleukine
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LVET	Low Volume Eye Test
MCP	monocyte chemotactic protein
MEM	minimum essential medium
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
ml	millilitre
mm	millimetre
mm^2	square millimetre
MMAS	modified maximum average score

mmHg	millimetres mercury
MMP	Matrix Metalloproteinase
µl	Microlitres
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form
NaOH	sodium hydroxide
nm	nanometres
pg	picogramm
pH	potentia hydrogenii
PMN	Polymorphonuclear leucocytes
PVC	polyvinyl chloride
®	rights reserved
resp.	respectively
RWTH	Rheinisch Westfälisch Technische Hochschule
sec	seconds
SLS	sodium lauryl sulphate
tab.	table
TCA	trichloroaceticacid
TIMP	Tissue Inhibitor of Metalloproteinase
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

Introduction

Animal Experiments in the cosmetic industry have been criticized for a long time and voices have been being raised against it for decades. Traditionally the Draize Test (see next chapter) has been used when questions about reactions of the different parts of the eye, especially the cornea, to chemicals had to be answered. To manage the balance between predictability of biological responses as good as possible and exposing living animals to as little harm as possible in the same time is a quite difficult task. Therefore multiple studies to test alternatives to the Draize Test have been performed. Both in vitro and in vivo testings were developed, each with its specific (dis-)advantages. Our aim was it to create a kind of experiment that does not depend on living animals but is able to show the living corneas' response to chemicals as near as possible to physiological conditions.

Models of Eye Irritation Testing

Draize-Test

For more than 60 years the Draize-Test has been using live rabbits to assess the potential of chemical substances to cause human ocular irritation after accidental exposure (Draize 1944; Maurer, Parker et al. 2002).

To carry out this test 100µl of the substance to be tested are instilled in the rabbit's subconjunctival sac followed by forced eye blink to let the chemical spread over the ocular surface. After defined time units the eyes are examined macroscopically for irritation with a penlight. The corneas, iris and conjunctivas are evaluated for different parameters first defined by Draize et al. (Draize 1944), i.e. for the cornea degree and area of opacity; for the iris increased prominence, congestion, swelling, circumcorneal injection, hemorrhage, gross tissue destruction and decreased pupillary reflex; for the conjunctiva redness, swelling and discharge (Jester JV 1998). Altogether there can be achieved a maximum average score as high as 110 as a combination of 80 for the cornea, 10 for the iris and 20 for the conjunctiva.

Different aspects regarding this test however have been criticized, i.e.(Marzulli and Simon 1971; Weil and Scala 1971; Heywood 1978; Freeberg 1986; Bruner 1992; Jester JV 1998):

- Subjectivity
- Lack of reproducibility
- Overestimation of human responses
- Use of live animals

Therefore attempts to replace the Draize Test have been made during the last years, so that nowadays normally the Low Volume Eye Test (Jester 2006; Secchi and Deligianni 2006) is used instead of the Draize Test. Methods to eliminate absolutely the need for in vivo testing have nevertheless yet not been realized, even if by now several approaches to this aim have been started. (Bruner, Kain et al. 1991; Balls, Botham et al. 1995; W.J.W.; ; Maurer, Parker et al. 2002)

Low Volume Eye Test (LVET)

Although being also a test using live animals the Low Volume Eye Test is an improvement on eye irritation testing compared with the Draize Test (Freeberg 1986).

10µl of the test substance, one tenth the volume instilled in the Draize Test, is placed directly on the rabbit's cornea. The following assessment regarding the eye irritation works on the same principles as in the Draize Test.

The two main advantages of the LVET are:

- Better correlation with human exposure
- Less stressful for the rabbits

The EYTEX method

The EYTEX™ system is an *in vitro* method for predicting in vivo ocular irritation via a target biomacromolecular approach (Gordon 1992; Gettings, Lordo et al. 1996). EYTEX™ uses changes of relevant macromolecules upon exposure to chemicals and formulations to predict in vivo irritancy and toxicity endpoints. These changes mimic the response of the protein in the cornea during corneal injury or ocular irritation. Quantification of the EYTEX™ method is based on measurement of changes in optical density of the matrix, the EYTEX™ reagent, which are sensitive to the conformation and

hydration of protein as well as the order in the matrix. These changes, which occur when the matrix is exposed to chemicals and formulations, can be used to predict in vivo ocular irritancy.The method has been extensively evaluated using chemicals and formulations from diverse classes and with different ranges of toxicity, representing various mechanisms of ocular toxicity. However there is a long list of chemicals which produce under- and overestimation resp. in comparison to the results of in vivo testing, i.e. the Draize Test, so that the significance of the EYTEX Method is limited (Bruner, Kain et al. 1991; Balls, Botham et al. 1995).

Red Blood Cell Hemolysis Test

This test measures the damage done to cell plasma membranes and/or the denaturation of membrane and other cellular proteins caused by chemical irritants. Such events can be correlated with the initial inflammatory response in tissue irritation and with changes in protein conformation, such as those that occur in opacification of the cornea.

The parameter for membrane damage is hemoglobin leaking from the red blood cells which is detected spectrophotometrically at 541 nm. Protein denaturation is measured by determing the reduction in absorbance at 541 nm with increasing concentrations of the test substance. All determinations are made relative to concurrent control sample, totally lysed with distilled water (Pape, Pfannenbecker et al. 1987; Pape WJ 1991; Lewis 1993; Pape, Maurer et al. 2001).

HET-CAM Assay (Hen's Egg Test Chorioallantoic Membrane)

In the HET-CAM Assay, first developed by Luebke (Luepke 1985) test materials are applied to the vascularized chorioallantoic membrane (CAM) of embryonated chicken eggs. The acute irritating effects on the small vessels and protein membranes of this soft tissue membrane are assumed to be similar to those induced by the same chemicals in the eye.

The score to describe the irritating properties of test materials consists of three end-points, i.e. hemorrhage, lysis and coagulation. Either a time point at each of the end-points is evaluated after application of the test material or the threshold concentration of appearance of the three endpoints is assessed, depending on the respective protocol used with the different test materials.

Bovine corneal opacity/permeability test (BCOP)

For the BCOP bovine corneas are prepared and put in special holders which consist of an anterior and a posterior chamber which are both filled with culture medium. The test material is applied to the anterior chamber of the holder and the corneas are exposed to it for one hour.

The endpoint of opacity is measured with an opacitometer which detects a change in light transmission passing through the cornea. Subsequently the medium is removed from both chambers in the holder and replaced by fresh medium and 1ml fluorescein solution in the posterior chamber. The corneas are then incubated in a horizontal position for 90 minutes at 37°C. After removing the medium the optical density is determined in a spectrophotometer at 490nm (Gautheron P. ; Gautheron, Mayordomo-Blanco et al. 1994; Gettings, Lordo et al. 1996).

Principles of the cornea’s anatomy and physiology

The cornea is the eye's outermost layer. It is the clear, dome-shaped surface that covers the front of the eye. Although the cornea is clear and seems to lack substance, it is actually a highly organized group of cells and proteins. Unlike most tissues in the body, the cornea contains no blood vessels to nourish or protect it against infection. Instead, the cornea receives its nourishment from the tears and aqueous humor that fills the chamber behind it. In the corneal limbus the avascular cornea is connected to the blood system. As mentioned above these vessels are not important for the nutrition of the cornea, but they release leucocytes an proteins that invade and release into the corneal stroma(Benninghoff 1994; Reim 1996).

The corneal tissue is arranged in five basic layers, each having an important function. These five layers are (Junqueira 1996):

- Epithelium:

  The epithelium is the cornea's outermost region, comprising about 10 percent of the tissue's thickness, i.e. about 550µm in the centre. It consists of about six to seven layers of non-keratinized stratified squamous epithelial cells. The epithelium functions primarily to block the passage of foreign material, such as dust, water, and bacteria into the eye and other layers of the cornea and to provide a smooth surface that absorbs oxygen and cell nutrients from tears, then distributes these nutrients to the rest of the cornea. The epithelium is filled with thousands of tiny nerve endings that make the cornea extremely sensitive to pain when rubbed or scratched. The part of the epithelium that serves as the foundation on which the epithelial cells anchor and organize themselves is called the basement membrane. The cornea’s stemmcells, that are responsible for regenereation of the epithelial and endothelial cell layers, are situated at the corneal limbus, the border of the cornea and sclera.

- Bowman's Layer (Lamina limitans anterior)

  The Bowman’s layer lies directly below the basement membrane of the epithelium. It is a transparent sheet of tissue and is composed of strong layered collagen fibres, it contains no cells.

- Stroma

  Beneath Bowman's layer is the stroma, which comprises about 90 percent of the cornea's thickness. It consists primarily of water (78 percent) and collagen (16

percent), and does not contain any blood vessels. There are few cells in the corneal stroma, which form a flattened connective tissue lying between the fibrous tissue lamellae which constitute the cornea. The keratocytes have branching processes that communicate with those of other keratocytes. The collagen gives the cornea its strength, elasticity, and form (Newsome, Gross et al. 1982). The collagen's unique shape, arrangement and spacing are essential in producing the cornea's light-conducting transparency.

- Descemet's Membrane (Lamina limitans posterior)

  Under the stroma is Descemet's membrane, a thin but strong sheet of tissue that serves as a protective barrier against infection and injuries. Descemet's membrane is composed of collagen fibers and is produced as basement membrane by the endothelial cells that lie below it. Descemet's membrane is regenerated readily after injury.

- Endothelium

  The endothelium is the extremely thin innermost layer of the cornea. It is build of about 2500/mm^2 monolayered hexagonal cells, varying with the age (Laule, Cable et al. 1978). These cells are essential in keeping the cornea clear. Normally, fluid leaks slowly from inside the eye into the middle corneal stroma. The endothelium's primary task is to pump this excess fluid out of the stroma (Dikstein and Maurice 1972; Maurice 1972; Kuang, Li et al. 2004). Without this pumping action, the stroma would swell with water, become hazy and ultimately opaque. In a healthy eye, a perfect balance is maintained between the fluid moving into the cornea and fluid being pumped out of the cornea. Once endothelium cells are destroyed by disease or trauma, they are lost forever as they do not regenerate. If too many endothelial cells are destroyed, corneal edema and blindness ensue.

Principals of corneal wound healing

Simple mechanical removal of the epithelium, i.e. abrasions without any toxic, physical oder chemical damage regenerate within 3-5 days, depending on the size of the defect (Reim M. 1997). Cell division and migration of the stem cells from the limbal region in the basal cell layer form a new superficial cell layer to cover the stroma (Kuwabara, Perkins et al. 1976; Thoft 1983). As soon as more complicated mechanisms such as chemical burn, heat etc. occur this process of corneal wound healing may be delayed and even sustainable impaired (Reim M. 1980). Mild alkali burns e.g. with 0,25mol NaOH destroy the superficial epithelium and penetrate the anterior stroma. That leads to dilation of conjunctival blood vessels, edema, exsudation of leucocytes in the conjunctiva and infiltration of PMNs to the corneal stroma within a few hours (Robb 1962; Reim M. 1980; Williams, Paterson et al. 1982). The regeneration of the epithelium lasts about 10 days in this case. Severe eye burns induce long-standing corneal epithelial defects and ulcerations and consequently lead to many problems in wound healing of the ocular surface. After having burnt the epithelium the agents leading to a severe eye burn penetrate further into the anterior chamber and damage iris and lens. At the interface between necrosis and surviving cells mediators of inflammation are released, such as prostaglandins, leucotriens, cytokines, interleukine (Bhattacherjee 1979). The epithelial and stromal cells become necrotic after severe burns, but the extracellularmatrix of epithelium and stroma is able to withstand a large extent chemical agents and heat. But alkaline hydrolysis of matrix proteins is induced by alkali burns and the resulting peptides are antigenic (Thiel 1988).

There are four stadiums of eye burn from mild to severe to distinguish (Kuckelkorn 1995):

Tab.1: Severity code of eye burns

Grade I°	**Grade II°**	**Grade III°**	**Grade IV°**
Erosio Hyperemia	Erosio Ischemia > 1/3 Chemosis	Erosio Ischemia > 1/2 Chemosis Corneal opacity	Erosio Ischemia > ¾ Deep corneal opacity Extended necrosis Fibrin on the iris
Regeneration	Recirculation Regeneration	Ulcus Vascularisation Scars	Proliferation Ulceration Persistent epithelial defects Cataract, Glaucoma, Scars

Overview of the examined Growthfactors/Cytokines

Fibroblast Growth Factor (FGF)

Fibroblast growth factors are a family of 23 growth factors that are essentially involved in angiogenesis (Stegmann 1998), wound healing, and embryonic development. The FGFs interact with cell-surface associated heparan sulfate proteoglycans and the extracellular domains of FGF cell surface receptors (FGFRs) to trigger receptor activation and biological responses (Ornitz and Itoh 2001). FGFs are key-players in the processes of wound healing, proliferation and differentiation of cells, particularly endothelial cells; they (especially FGF-1) promote angiogenesis.

Vascular endothelial growth factor

The vascular endothelial growth factor (VEGF) is a sub-family of growth factors, more specifically of platelet-derived growth factor family of cystine-knot growth factors. Both the vasculogenesis (the de novo formation of the embryonic circulatory system) and the angiogenesis (the growth of blood vessels from pre-existing vasculature) are among the tasks of these signaling proteins (Ferrara ; Patan 2004). As its name implies, VEGF activity has been mostly studied on cells of the vascular endothelium, although it does have effects on a number of other cell types (e.g. stimulation monocyte/macrophage migration, neurons, cancer cells, kidney epithelial cells). In vitro, VEGF has been shown to stimulate endothelial cell mitogenesis and cell migration. VEGF is also a vasodilator and increases microvascular permeability and was originally referred to as vascular permeability factor (Ribatti 2005).

Tumor Necrosis Factor-α

TNF-α (formerly known as cachexin or cachectin (Beutler, Greenwald et al. 1985)) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that all stimulate the acute phase reaction. TNF causes apoptotic cell death, cellular proliferation, differentiation, inflammation, tumorigenesis, and viral replication. TNF's primary role is in the regulation of immune cells (Chen and Goeddel 2002).

Dysregulation and, in particular, overproduction of TNF have been implicated in a variety of human diseases, as well as cancer (Gaur and Aggarwal 2003).

Macrophage Inflammatory Protein-1 β

Macrophage Inflammatory Proteins (MIP) belong to the family of chemotactic cytokines known as chemokines. In humans, there are two major forms, MIP-1α and MIP-1β that are now officially named CCL3 and CCL4 respectively. Both are major factors produced by many cells, particularly macrophages, dendritic cells, and lymphocytes. They activate human granulocytes (neutrophils, eosinophils and basophils) which can lead to acute neutrophilic inflammation. They also induce the synthesis and release of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α from fibroblasts and macrophages (Maurer 2004).

Monocyte Chemotactic Protein-1

Monocyte chemotactic protein-1 (MCP-1) is a small cytokine belonging to the CC chemokine family that is also known as Chemokine (C-C motif) ligand 2 (CCL2). MCP-1 recruits monocytes, memory T cells, and dendritic cells to sites of tissue injury and infection (Carr, Roth et al. 1994). MCP-1 causes the degranulation of basophils and mast cells, an effect potentiated by pre-treatment with IL-3 and other cytokines (Conti, Boucher et al. 1995).

Interleukin-1α/1 β

Both IL-1α and IL-1β are produced by macrophages, monocytes and dendritic cells. They form an important part of the inflammatory response of the body against infection (Dinarello 1994). These cytokines increase the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes to sites of infection and re-set the hypothalamus thermoregulatory center, leading to an increased body temperature which expresses itself as fever (Boraschi, Villa et al. 1990). IL-1 is therefore called an endogenous pyrogen. IL-1 is also important in the regulation of hematopoiesis. For the most part, these two forms of IL-1 bind to the same cellular receptor. This receptor is composed of two related, but non-identical, subunits that transmit intracellular signals via a

pathway that is mostly shared with certain other receptors. IL-1 alpha is known to induce apoptosis of corneal endothelium. IL-1 alpha also increased NO production in the cornea. Besides, it is a strong inducer of MMP and TIMP in the cornea and a Promotor of corneal edema and inflammation (Yamada, Dana et al. 2003). Last not least, very high levels of IL-1 alpha were found in human corneas obtained from keratoplastics in severe inflammatory diseases (Becker, Salla et al. 1995).

Interleukin-8

Interleukin-8 (IL-8) is a chemokine produced by macrophages and other cell types such as epithelial cells. It is also synthesized by endothelial cells, which store IL-8 in their storage vesicles, the Weibel-Palade bodies (Wolff, Burns et al. 1998). This chemokine is one of the major mediators of the inflammatory response and is secreted by several cell types. It functions as a chemoattractant, and is also a potent angiogenic factor. Primary function of IL-8 is the induction of chemotaxis in its target cells (e.g. neutrophil granulocytes) (Baggiolini and Clark-Lewis 1992). In neutrophils series of cell-physiological responses required for migration and its target function phagocytosis are also induced like increase of intracellular Ca2+, exocytosis (e.g. histamine release), respiratory burst. IL-8's primary function is to recruit neutrophils to phagocytose the antigen which trigger the antigen pattern toll-like receptors.When first encountering an antigen, the primary cells to encounter it are the macrophages who phagocytose the particle. Upon processing, they release chemokines to signal other immune cells to come in to the site of inflammation. IL-8 is one such chemokine. It serves as a chemical signal that attracts neutrophils at the site of inflammation, and therefore is also known as Neutrophil Chemotactic Factor.

Interleukin-10

Interleukin-10 (IL-10), also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. This cytokine is produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of other cytokines, MHC class II antigens, and costimulatory molecules on macrophages (Moore, de Waal Malefyt et al. 2001). It also enhances B cell survival, proliferation, and antibody

production. It is capable of inhibiting synthesis of pro-inflammatory cytokines made by cells such as macrophages and the Type 1 T helper cells. IL-10 also displays potent abilities to suppress the antigen presentation capactiy of antigen presenting cells. However, it is also stimulatory towards certain T cells, mast cells and B cells (Groux and Cottrez 2003).

Materials and Methods

Rabbit eyes

Since our major task was to replace eye irritation tests using live animals we obtained rabbit heads from a nearby slaughtery for food supply (H.-J. Lammers, Dipl.-Ing.agr., Pützhof, Dompfaffstr. 34, 53881 Euskirchen). Within four hours after the animals' death due to carbondioxide gas the heads were delivered. The eyes were rinsed with Polyspectran® eye drops (Alcon) at the slaughtery then closed and stuck up with tape to avoid the corneas to suffer from drying.

Enucleation/Preparation

After having removed the tape from the eyes we rinsed the eyes once again with Polyspectran® eye drops (Alcon), then we performed the enucleation under a laminar-flow bench for sterile working. Further on the eyeball was laid down with the corneal side downwards on a specially constructed block made of silicone with a concave mould and a central opening of 12mm in diameter. We started the preparation by opening the eye bulb on the optic nerve head, cutting eight slices radially near to the eye equator and pinning them with needles onto the block. Carefully to cause no damage on the cornea, especially on the endothelium, the vitreous, iris, lens, chorioidea and retina were removed. After having rinsed the remaining cornea and the adjoining sclera with a solution of Ringer-Lactate (Fresenius) and antibiotics/-mykotics (piperacillin, amikacin, nystatin) we transferred the cornea into the perfusion chamber.

Enucleation set:

- A pair of conjunctival scissors
- A pair of bulbus pincers
- 1 squint hook
- A pair of opticus-scissors

Preparation set:

- 1 preparation block with a silicone ring
- 4 sterile cannulas to fix the sclera on the preparation block
- 2 colobri pincers
- 2 surgical micro pincers

- 2 scissors for conjunctiva or other tissues

Perfusion chamber

The perfusion chamber consisted of two blocks made of plexiglas with a hole in the size of the cornea in the middle as the main parts. Between these blocks the cornea was placed with the epithelial part located upwards, fixed by a triangle shaped ring pressing the sclera to the upper parts of the blocks. In this way the epithelium was exposed to the air, the endothelium to the culturing medium flowing through the inner parts of the chamber. The chamber was then connected to three-way stopcocks at the in- and outlet to guarantee the supply and the outflow of the culturing medium which was filled in afterwards. The volume of each chamber including the Luer lock connectors of the chambers was about 0.5 to 0.9 ml, depending on the corneal curvature.

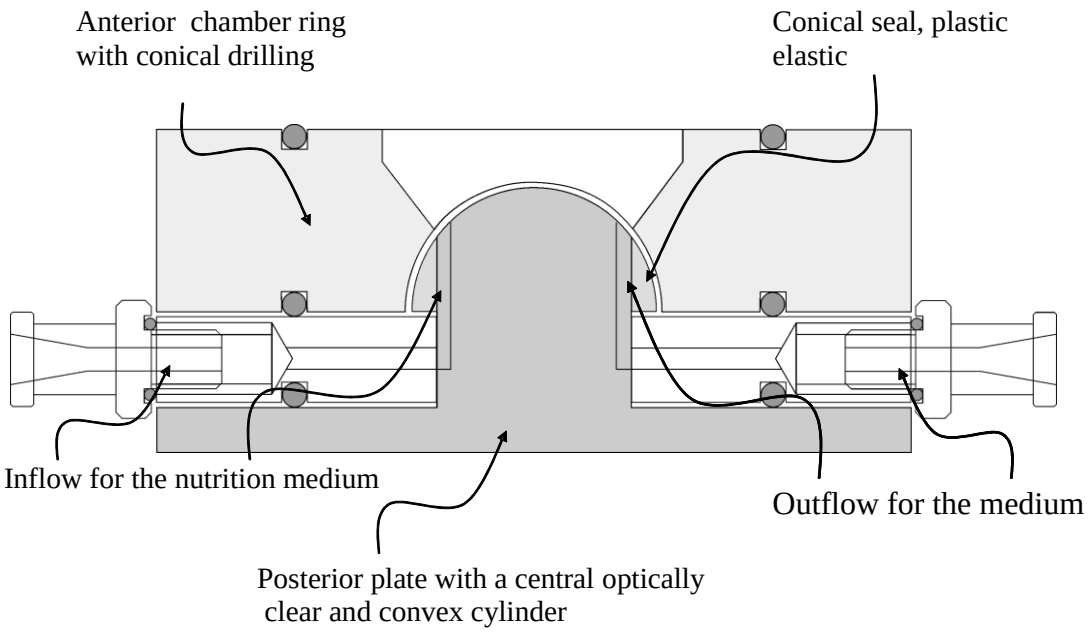


Fig. 1: Perfusion chamber 1

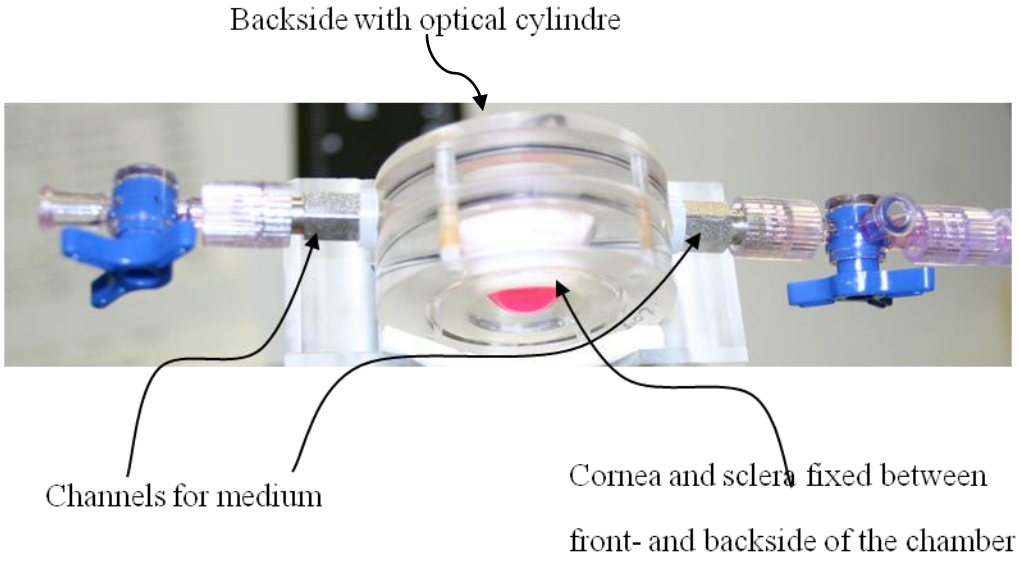


Fig. 2: Perfusion chamber 2

Culturing medium

The medium we used for the corneas' nutrition is similar to the Minimum Essential Medium (MEM) used for corneal organ culture, leaving out the fetal calf serum to exclude biochemical factors such as growth factors. The medium contained antibiotics (piperacillin, amikacin, nystatin) and was self-produced.

Experimental setup

To process more corneas, i.e. to achieve more data at the same time, the experimental setup (see fig. 3) was in a way that we were able to handle eight chambers simultaneously. They were put in an incubator with a constant temperature of 31°C. As the eye blinking to moisten the epithelium was not possible we used 100% air humidity in the incubator.

The medium distribution to the chambers was guaranteed by a micro-pump with a 16 channel parallel drive (ISMATEC High Precision Tubing Pump with planetary drive, IPC 16 channels ISM 930) that was installed in the outflow of the chambers. Since the physiological flow rate within the eye's anterior chamber is about 5 to 15 µl, we decided to keep the system's flow rate constantly at 10 µl, so that a total fluid exchange in

the artificial anterior chamber was realized within 50 to 90 minutes depending on the chamber's volume. We installed tubings leading from the pump to a waste container outside the breeder.

For obtaining a constant pressure of 6 mmHg in the chambers which has been proved in earlier testings to be best to avoid corneal turbidity and to maintain the corneal shape without leakage through the clamping mechanism we put a medium reservoir (50 ml Falcon® tube) with a level control at the height of approximately 9.8 cm above the inner component of the chambers in the breeder.

This reservoir was filled by two perfusor pumps in which four 50 ml syringes were clamped into, each draining with a constant flow of 1.5 ml per hour. As for the eight chambers, which drained each 0.6 ml per hour (10 µl per minute), only 4.8 ml were needed instead of 6 ml, the surplus of medium in the reservoir was pumped off by an additional connection to the pump. By constructing it this way we avoided any chance of having too less medium in the reservoir to fill the chambers due to eventually occuring inaccurate draining.

By trying to obtain a constant pH of 7.4 in the medium it turned out that the tubings that connected the chambers with the reservoir lost carbondioxid gas so that the pH of the medium arriving the chambers after the passage through the tubings was much higher (about 7.8) than wished. Therefore we had the medium in the reservoir to be perfused with additional carbondioxid gas.

This whole system with eight parallel perfusion chambers was composed of:

- 4 50ml Braun Perfusor®-Syringes with 25 mm Syringe Filters with 0.2µm Supor® membrane connected by
- 4 Braun Perfusor®-tubings PE (length: 50 cm, inner diameter: 1 mm) with
- 3 multidirectional stopcocks Braun Discofix®, disemboguing in
- 1 Braun Perfusor®-tubing PE (length: 200 cm, inner diameter: 1 mm) leading to
- 50 ml Falcon® tube as reservoir for the medium inside the breeding unit
- 1 canula stuck at the bottom of the Falcon® tube connected with
- 9 multidirectional stopcocks Braun Discofix®, one in the middle, four to each side, each one conjoined with
- 1 Braun Perfusor®-tubing PE (length: 150 cm, inner diameter: 1 mm), leading to the chambers (in-/outflow with Discofixes®). Behind the chambers:

- 8 Braun Perfusor®-tubings PE (length: 50 cm, inner diameter: 1 mm), connected with
- 8 multidirectional stopcocks Braun Discofix®, combined with
- 8 Braun Perfusor®-tubings PVC (length: 150 cm, inner diameter: 1 mm),
- 8 pumping tubes (ISMATEC Tygon LFL SC0417, inner diameter: 0.64mm), passing through the pump (ISMATEC IP-N 16), which warrants a constant flow of 10µl/min, and leading over
- 8 Braun Perfusor®-tubings PVC (length: 50cm, inner diameter: 1 mm) to
- 8 50ml Falcon® tubes as waste bottles
- At the 50ml Falcon® tube as reservoir a Braun Perfusor® tubing PVC (length 200cm, inner diameter: 1 mm) was installed as spillway leading into
- 1 pumping tube (ISMATEC Tygon LFL SC0417, inner diameter: 0.64mm), passing through the pump (ISMATEC IP-N 16) and leading over
- 1 Braun Perfusor®-tubings PVC (length: 50cm, inner diameter: 1 mm) to
- 1 50ml Falcon® tubes as waste bottle

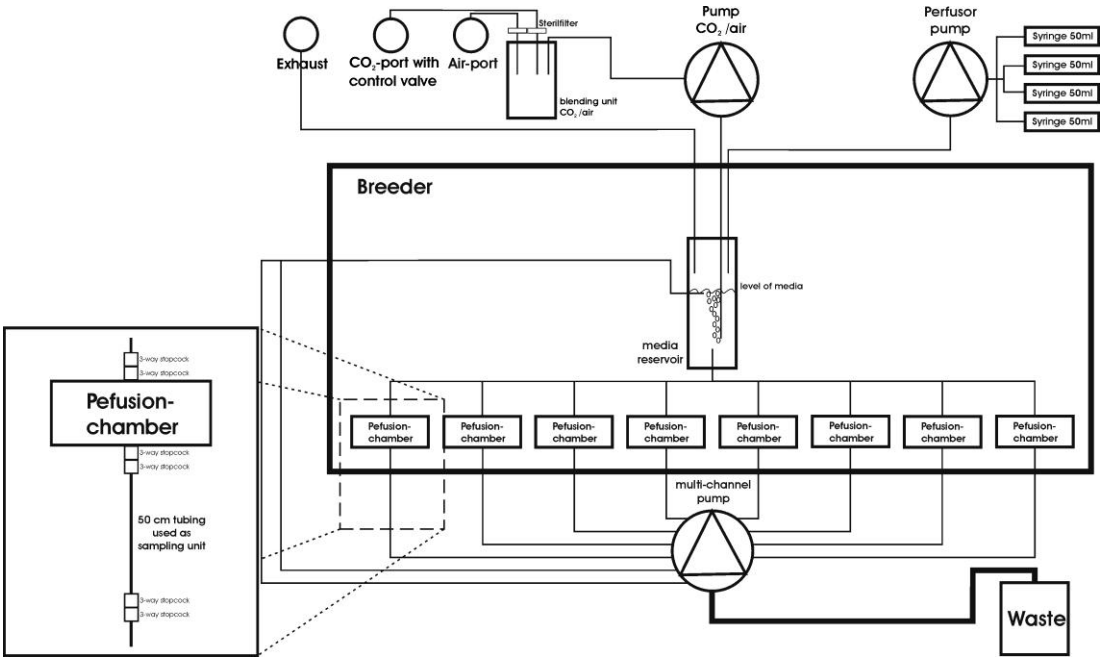


Fig. 3: experimental setup

Specimen sampling and measurements

After having processed the corneas and having connected them to the tubings in the incubator we left them without any treatment for the next nine hours. Then we started with the first samplings of the medium specimen to perform measurements of glucose consumption and lactate production, pH, LDH, and cytokine mediator release.

Handling

We were interested in medium samples that were in contact with the endothelial as well as the epithelial side of the cornea, so that we performed two different styles of medium sampling. The endothelial one was done by taking the medium that was in the 50cm-tubings between the chamber and the pump. The content of these tubings was about 0.7 ml each. For taking the epithelial samples we exposed the corneas for 15 minutes with one ml MEM and removed it afterwards with a syringe. This procedure had the desirable side effect that the cellular debris, which accumulates on the corneal surface and is normally, under physiological conditions removed by eye blinkings, was at least partially cleared away.

This procedure was repeated at 15, 19, and 36 hours after preparation before doing any harm to the corneas. After the chemical and mechanical treatment respectively we did the measurements hourly for the first eight hours, then once a day for seven days.

pH, glucose, lactate measurements:

These parameters were measured by means of an automatic micro-volume analyser from Bayer®: Rapid lab 860®. The used volume did not exceed 100 µl for all three analyses.

LDH analysis:

We analysed LDH release in both media by means of an NADP-NADPH transfer of lactatic acid to pyruvate kinetic assay.

Cytokines and growth factors:

These analyses were performed from pooled samples with the Luminex system using the R&D Fluorokine MAP assays in the laboratory of the Institute for Diabetological Research at Düsseldorf. The assays were proven to be cross reactive with LPS activated rabbit serum. The following antibodies from R & D proved to be positive for LPS stimulated rabbit blood cells: IL-1-alpha, IL-1-beta, IL-8, IL-10, MCP, TNF-alpha, MIP1-

beta, FGF and VEGF-165. Like LPS stimulated rabbit blood cells experimental samples should exhibit proportional responses to the analytical antibodies. Therefore, it was concluded, that cross reactivity of human antibodies could be used to measure cytokines from experiments with ex vivo rabbit corneas. Assays were accepted only, when values were found to be within the ranges of reliability given in the technical data sheets according to the specific parameter. The used antibodies were provided by R&D Systems as part of a multiplexed assay (containing IL1 alpha (LUH200), IL1-beta(LUH201), IL2 (LUH202), IL 8 (LUH208), , VEGF-165 (LUH293), MIP1-beta (LUH271), bFGF (LUH233)).

Photographical documentation

Each day we took a full frame digital image of the cornea within the chamber from the top (Casio 5700, Canon EOS D500) The camera was installed upside down looking on the centred corneas. Photographs were taken before, directly after and once a day after the exposure to chemical injuries. All corneas were stained with fluoresceine on day 6 or 7. The greenish reflex of the fluoresceine was used to judge the epithelial integrity.
Additionally pictures of the endothelium and epithelium were taken on the day of preparation and the final day with Diskus/Leica Invertoscope System.
Photograhs of the endothelium were taken with an Apochromatic microscope from Leica, which was available for the testings of Tomadol 45-7 3%, all concentrations of TCA and SLS and those of H_2O_2 (1,5%, 6%, 12%) and NaOH (0,2mol, 0,4mol, 0,8mol)

Microbiological examination

We performed microbiological tests on the corneas at the end of any experiment. We took a swab of the cornea's epithelium on blood agar plate as well as samples of the medium. All controls of sterility were done in the Department of Microbiology RWTH Aachen

Exposure with chemicals

All corneas were exposed with a Low Volume Eye irritation system consisting of an upsite down positioned applanation Tonometer from Haag Streit. On the microbalance

measuring head a contact lens of 7.5mm inner curvature was glued on and in this contact lens a volume of 10 µl of the test substance was applied. With a centred positioning stative the chamber was upside down positioned and by means of downpositionning of the cornea the cornea touched the contact lens. The cornea was exposed for 30 seconds to the substance. After exposure the cornea was rinsed for another 30 sec. with medium.

Chemical substances

The chemicals we took were chosen on behalf of their chemical differences in action and their wide spread use. The references of the used concentrations were taken from the MMAS scales being published in the ECETOC database

Description of the set of chemicals:

Tomadol E 14-5:

3 % Cationic detergent: Chemical Description: poly (5) oxyethylene isodecyloxypropylamine; Family: Ethoxylated Amine Net Weight: 440
© 2003 Tomah Products
Used in one concentration of 3 % on 16 corneas

Tomadol E 45-7:

Tomadol E 45-7: Non-ionic NON3: Alkyl E7 (average) ethoxylate is a nonionic surfactant made from linear C14-15 alcohol with 7 moles (average) of ethylene oxide. Tomadol 45-7 has an HLB of 11.6 and is typically used as the sole surfactant or in combination with other nonionic surfactants. CTFA Name: C14-15 Pareth-7; Chemical Description:
poly (7) oxyethylene C14-15 alcohol Family: Alcohol Ethoxylate
© 2003 Tomah Products
Used in one concentration of 10 % on 16 corneas

NaOH:

2 mol weight 40 g / Mol, PHYSICAL STATE White, deliquescent pellets or flakes, MELTING POINT 318° C, BOILING POINT 1390° C, SPECIFIC GRAVITY 2.13 g/ml; SOLUBILITY IN WATER pH 13 - 14 (0.5% sol.); used in 4 concentra-

tions: 0.2 (n=3 corneas), 0.4 (n=3 corneas), 0.8 (n=3 corneas) and 2 molar (n=16 corneas)

H_2O_2:

Hydrogen Peroxide is a strong oxidizing agent and a weak acid in water solution. PHYSICAL STATE Clear, colorless liquid, MELTING POINT -11° C (90%), -39° C (70%), BOILING POINT 141° C (90%), 125° C (70%) SPECIFIC GRAVITY 1.4 g/ml (90%), 1.3 g/ml (70%) SOLUBILITY IN WATER Infinitely soluble; pH 1.3 (70%); used in 4 concentrations: 15% (n=3 corneas), 3% (n=16 corneas), 6% (n=3 corneas) and 12 % (n=3 corneas)

Controls:

Untouched corneas within the culturing system n=16 corneas
Mechanical abrasion n=16 corneas

Morphological Analysis of the corneas

In a scoring system, that presented a modification of the score for epithelial damage used by Draize, we evaluated images of all corneas being processed within the system exposed to known concentrations of the substances. The endothelial cells were judged using a scoring pattern being developed during examinations of multiple images of the specular microscope showing alterations of the endothelium of the ex vivo cornea. Later, at the end of the experiment, the artificial anterior chamber was filled with trypan blue staining cells with severe defects of the cellular membranes indicating cell death. Cells stained with trypan blue were regarded to be death following the guidelines of the European cornea bank association.

Analyses of photographs

At the end of all culturing experiments including normal corneas, untreated ex vivo controls, the groups of n=16 corneas each treated with Tomadol E14-5, Tomadol 45-7, NaOH 2mol and Hydrogenperoxide, abrasion, and the dose response experiments, the files of the taken images as desdribed above were sorted by quality of photographs and in a blinded manner presented to Prof. Schrage who scored all photographs (n=720 evaluations) using up to 2 to 3 photographs per evaluated day and cornea. All MMAS scores were calculated 2 with the following formula: MMAS = 5 x (grade of area of epithelial defect * grade of opacity)

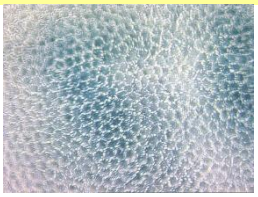
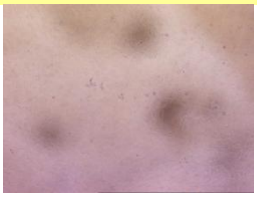
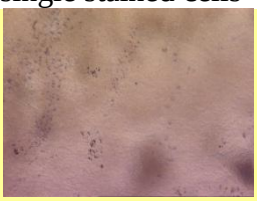
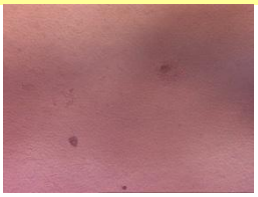
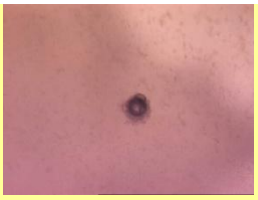
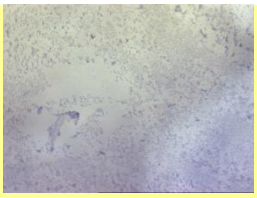
Epithelial Score

Tab. 2: Grading of epithelial damage

Draize 1944, Friedenwald 1944, OECD 1987	**Schrage et al 2005**	
No opacity	clear	0
Scattered or diffuse area, details of iris slightly obscured	Increased scattering hardly to see	1
Easily discernible translucent areas, details of iris slightly obscured	Easily to see opacities of the corneas	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	Clear opacities with low visual acces towards the inner chamber, whitish aspect	3
Opaque iris not visible	opaque	4
Area involved	No area involed	0
	< 10%	1
	< 25%	2
	< 50%	3
	> 50%	4
Epithelialisation visible with slit-lamp and fluoresceine stain	Epithelium present > 90%	0
	Epithelium present > 75%	1
	Epithelium present > 50%	2
	Epithelium present > 25%	3
	Epithelium present > 0%	4

Endothelial score

Tab. 3: Grading of endothelium damage

Unstained images	Trypan stained images	Score
 endothelium smooth dense	 no stained cells	0
 endothelium swollen	 single stained cells	1
 endothelim irregular	 Stripes of stained cells inbetween intact endothelium	2
 endothelium with gaps	 Conglomerates of stained cells without other endothelium	3
 no endothelium	 No endothelium or complete necrosis	4

Quantity of analysed corneas (epithelium)

Tab. 4: Photographs epithelial side

	Day 0	Day 4	Day 6/7 (fluo)	Day 6/7 (unstained)
NaOH 0,2mol	8	8	8	8
NaOH 0,4mol	3	3	3	3
NaOH 0,8mol	3	3	3	3
NaOH 2,0mol	16	16	16	16
H_2O_2 1,5%	3	3	3	3
H_2O_2 3,0%	16	16	16	16
H_2O_2 6,0%	3	3	3	3
H_2O_2 12,0%	3	3	3	3
Tomadol 45-7 3%	16	16	16	16
Tomadol E14-5 10%	16	16	16	16
SLS 1,5%	3	3	3	3
SLS 3,0%	3	3	3	3
SLS 15%	3	3	3	3
SLS 30%	3	3	3	3
TCA 3,0%	3	3	3	3
TCA 6,0%	3	3	3	3
TCA 12,0%	3	3	3	3
TCA 30,0%	3	3	3	3
Abrasion	16	16	16	16
No trauma	4	4	4	4

Quantity of analysed corneas (endothelium)

Tab. 5: Photographs endothelial side

	Day 2	Day 4	Day 6/7 (fluo)	Day 6/7 (unstained)
NaOH 0,2mol	3	3	3	3
NaOH 0,4mol	3	3	3	3
NaOH 0,8mol	3	3	3	3
H_2O_2 1,5%	3	3	3	3
H_2O_2 6,0%	3	3	3	3
H_2O_2 12,0%	3	3	3	3
Tomadol 45-7 3%	8	8	8	8
SLS 1,5%	3	3	3	3
SLS 3,0%	3	3	3	3
SLS 15%	3	3	3	3
SLS 30%	3	3	3	3
TCA 3,0%	3	3	3	3
TCA 6,0%	3	3	3	3
TCA 12,0%	3	3	3	3
TCA 30,0%	3	3	3	3
No trauma	4	4	4	4

Results

Morphological evaluation

MMAS calculated from epithelial photographs of the ex vivo corneas

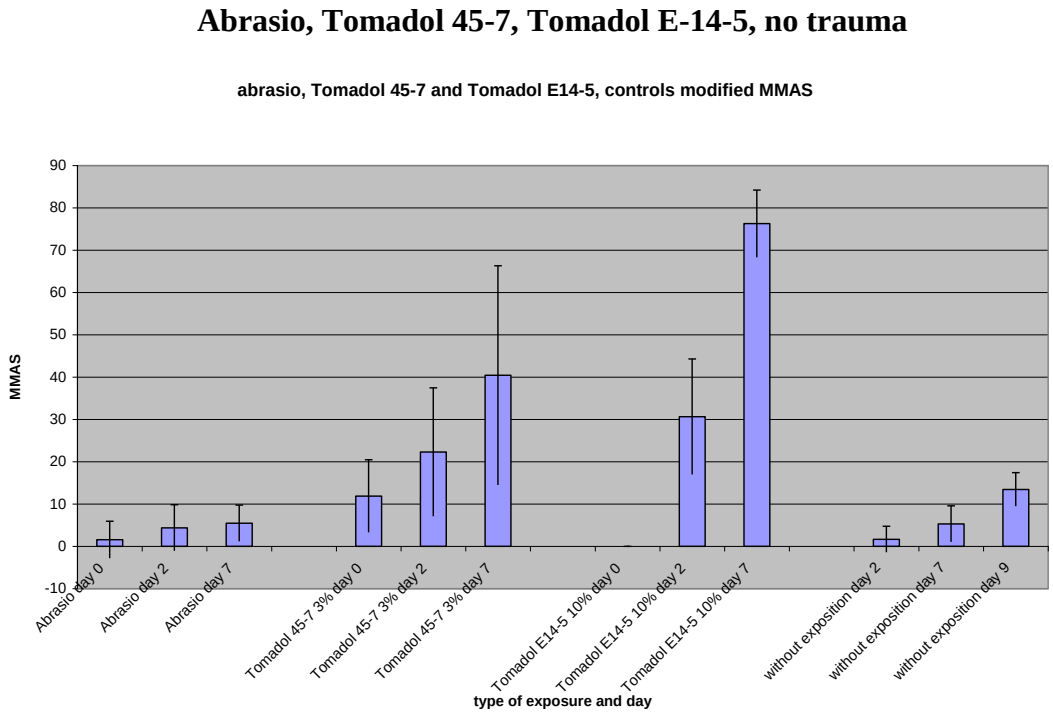


Fig. 4: Calculated MMAS from ex vivo corneas following experiments with mechanical abrasio (n=16), exposure to Tomadol 45-7 (n=16), Tomadol E14-5 (n=16), and the control group (n=4) on days 0, 2, 7 and 2, 7, 9 respectively.
Abscissa: experimental groups/types of exposure, time points of evaluation; ordinate: mean values and standard deviations of the MMAS scores

The MMAS scores of those ex vivo corneas with an abrasion of the epithelium done on them and those with no exposition to any trauma scarcely showed increasing values (fig. 4). After the application of Tomadol 45-7 and E14-5 resp. the calculated MMAS scores increased from day 0 to day 2 and further on to day 7 according to the rising worsening of the clinical picture.

Hydrogen Peroxide (H_2O_2)

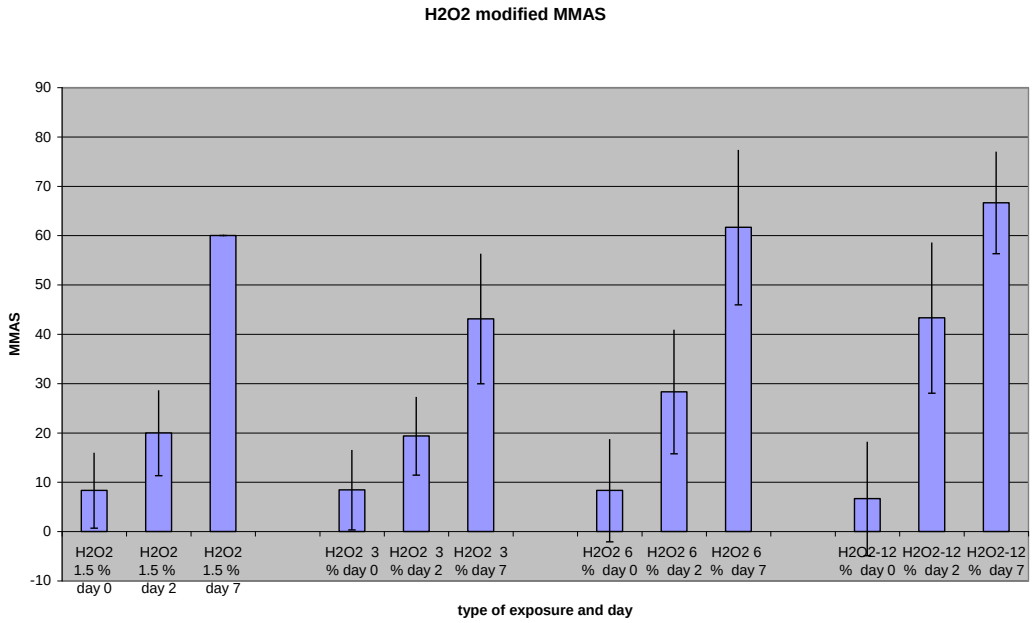


Fig. 5: Calculated MMAS from ex vivo corneas on day 0 directly and 2 and 7 days after exposure to hydrogene peroxide (H2O2) 1.5 %, 3 % (n=16 column), 6 % and 12 % (n=3 each column). Abscissa: concentrations applied and time points of evaluation; ordinate: mean values and standard deviations of the MMAS

The exposure to H_2O_2 (Fig. 5) showed an increase in the MMAS responses with time up to day 7 as well as with the concentrations of H_2O_2 applied, esp. on day 2. During exposure to the higher concentrations 6 % and 12 % H_2O_2, bubbles appeared on the corneal surface. This was certainly due to the immediate decontamination of hydrogenperoxide by endogenous superoxide-dismutase and catalase present in epithelial cells. Therefore the amount of H_2O_2 and consequently the dose reaching the corneal stroma might have been modified. However, the MMAS scores calculated from exposure characteristics on days 2 showed a good dose response of MMAS to increasing H_2O_2 concentrations from 3% over 6% up to 12%.

Trichloro-acetic acid (TCA)

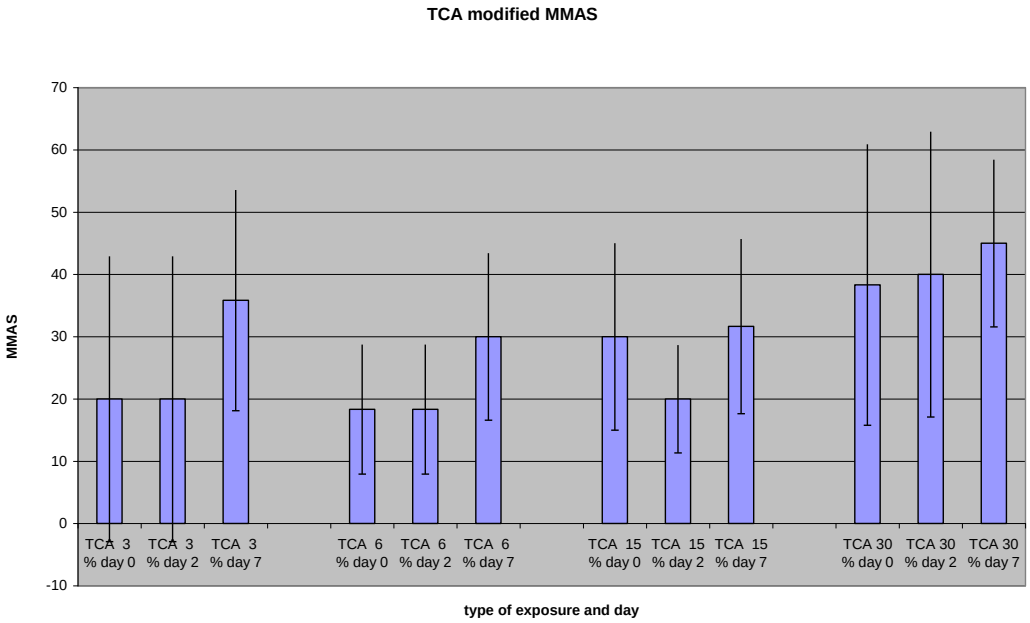


Fig. 6: Calculated MMAS from ex vivo corneas following exposure to trichloro-acetic acid (TCA) 3 %, 6%, 15% and 30 %, on days 0 (directly after exposure), day 2 and day 7 (each column n=3). Abscissa: experimental groups/types of exposure and time points of evaluation; ordinate: mean values and standard deviations of the MMAS scores

Application of TCA on the surface of the ex vivo corneas (Fig. 6) showed rather high calculated MMAS scores at all time points after exposure which slightly increased on higher concentrations of TCA. But because of the rather high standard deviations, the whole diagram of Fig. 6 does not demonstrate clear relationship between MMAS scores and TCA concentrations or time after exposure.

Sodium-Laureylsulfat (SLS)

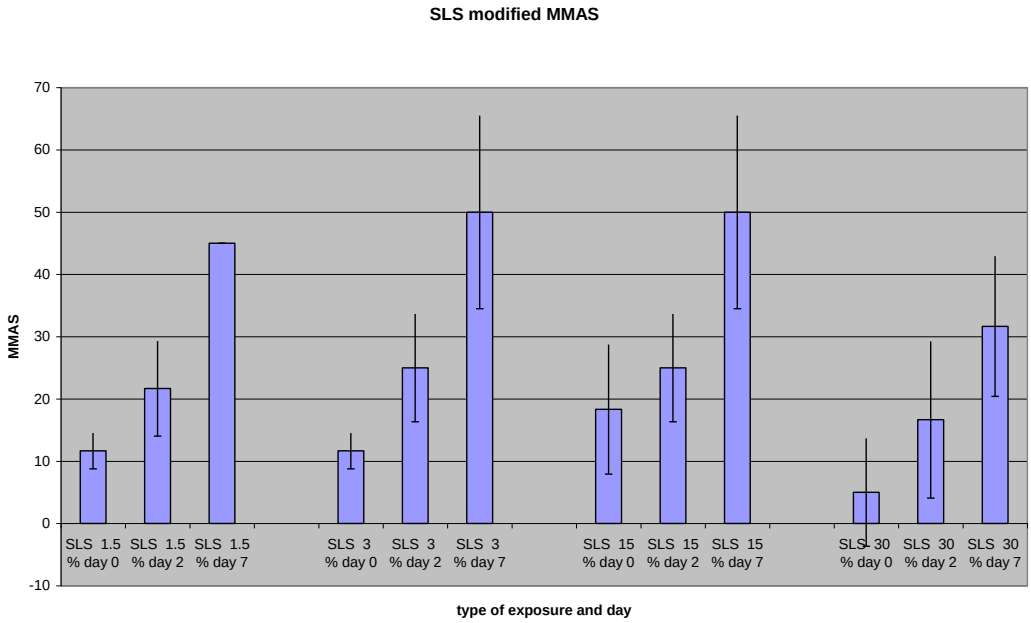


Fig. 7: Calculated MMAS from ex vivo corneas following exposure to sodium laureylsulfate (SLS) 1.5%, 3 %, 15% and 30 %, on days 0 (directly after exposure), day 2 and day 7 (each column n=3). Abscissa: experimental groups /type of exposure and time points of evaluation; ordinate: mean values and standard deviations of the MMAS

In the experiments with SLS (Fig. 7), the calculated MMAS scores of the ex vivo corneas increased with time after the exposure showing that the damage to the cornea increased with time. Dose responses were not detected. The MMAS scores calculated from the experiments with the 30 % SLS group were less severe than the others.

Sodium Hydroxide (NaOH)

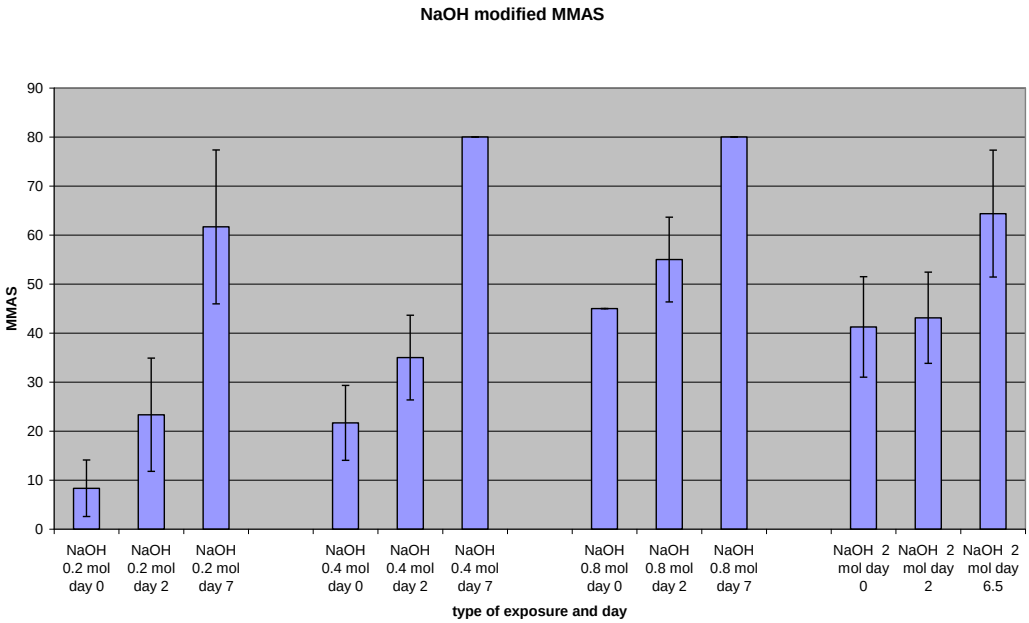


Fig. 8: Calculated MMAS from ex vivo corneas following exposure to Sodium hydroxide (NaOH) 0.2 mol, 0.4 mol, 0.8 mol (each n=3) and 2 mol (n=16) , on days 0 (directly after exposure), day 2 and day 7.

Abscissa: experimental groups/types of exposure and timepoints of evaluation; ordinate: mean values and standard deviations of the MMAS scores

The exposure of the ex vivo cornea to NaOH (Fig. 8) showed calculated MMAS scores compatible with dose responses on days zero and two from 0,2 mol to 0,4 mol and further to 0,8 mol. On day seven after exposure, the damage of the ex vivo corneas seemed to be at maximum and did not result in higher calculated MMAS scores.

Epithelial healing

Epithelial healing has been considered as an essential part of the recovery. Therefore, the healing of the epithelium on the ex vivo corneas, clinically visible with the slitlamp microscope, was investigated in the same set of experiments as described in the preceeding chapter. The areas of the ex vivo corneas covered with epithelium were measured in the experiments following mechanical abrasion, and with exposure to 2 mol NaOH, 3% H_2O_2, 3% Tomadol 45-7, 10% Tomadol E 14-5. In each of these groups n=16 separate ex vivo corneas were included. The results of epithelial healing are presented in Fig. 9 and 10. The corresponding calculated MMAS-scores of the same ex vivo corneas were shown already in Fig. 4 and partly in Fig. 5 and 8.

The epithelial healing was also assessed in the dose response experiments (n=3 each) after exposure to various concentrations of NaOH, H_2O_2, TCA and SLS. These results are presented separately in the following diagrams 11 to 14. - The corresponding MMAS scores of the same ex vivo corneas were already given in Fig. 5-8.

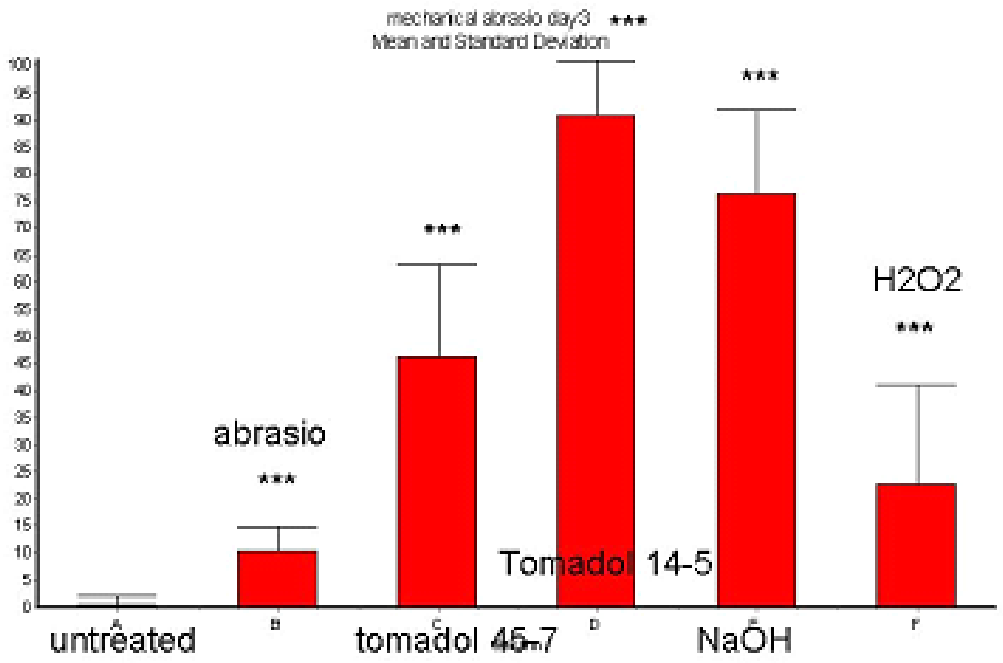


Fig. 9: Epithelial defects of ex vivo corneas on day 3 after mechanical abrasion and exposures to 3% Tomadol 45-7, 10% Tomadol E14-5, 2 mol NaOH and 3% H2O2. (each n=16)

Abscissa: experimental groups; ordinate: size of epithelial defects [%] of the total corneal surface, mean values and standard deviations of epithelial defects

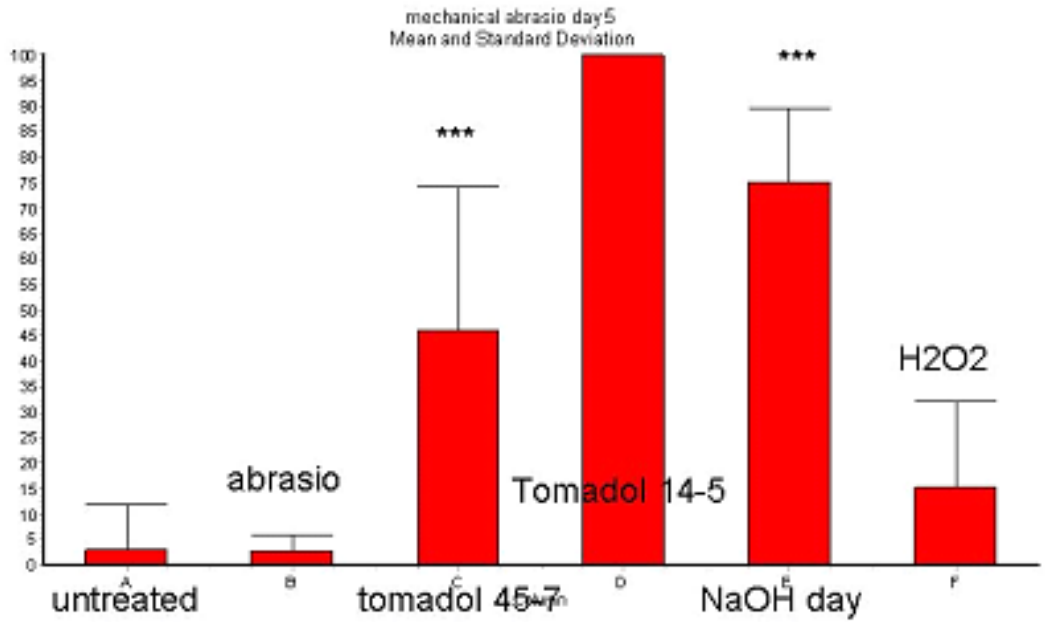


Fig. 10: Epithelial defects of ex vivo corneas on day 5 after mechanical abrasion and exposures to 3% Tomadol 45-7, 10% Tomadol E14-5, 2 mol NaOH and 3% H2O2. (each n=16)
Abscissa: experimental groups; ordinate: size of epithelial defects [%] of the total corneal surface, mean values and standard deviations of epithelial defects

The untreated corneas did not show any epithelial defects after the first three days of incubation (Fig. 9). However, as seen in the evaluation on day 5, on the bar for the untreated corneas, a slight fluoresceine positive defect was recorded (Fig. 10). This is referred to very small irregular fluoresceine staining spots in the center of the cornea, which appeared after several days due to surface drying. This staining results from desquamation of superficial cells, not from through and through epithelial defects.

The abrasion of the epithelium resulted in a total defect of the epithelium. Three days later, as seen in Fig. 9, the epithelium of the ex vivo cornea was healed down to a remaining erosion of about 10% of the total corneal surface. After five days, the epithelial defect was as good as closed (fig. 10).

Healing of the epithelium on the ex vivo corneas was delayed to various extent after exposure to the Tomadols, NaOH and H_2O_2. It should be mentioned, that the chemicals applied in these experiments were rather toxic and applied in very high concentrations. Quantitatively different responses to various chemicals/traumas could be demonstrated: mechanically removed epithelium healed to almost zero defects, after exposure to H_2O_2 the epithelial defects were reduced to about 10% on day 5 (fig. 10). Exposure to 2 mol NaOH and to Tomadol 45-7 left unchanged epithelial defects of 75% and 45%, respectively, and with Tomadol E 14-5 the 90% defect enlarged to 100% (fig. 10).

Dose response in epithelial healing

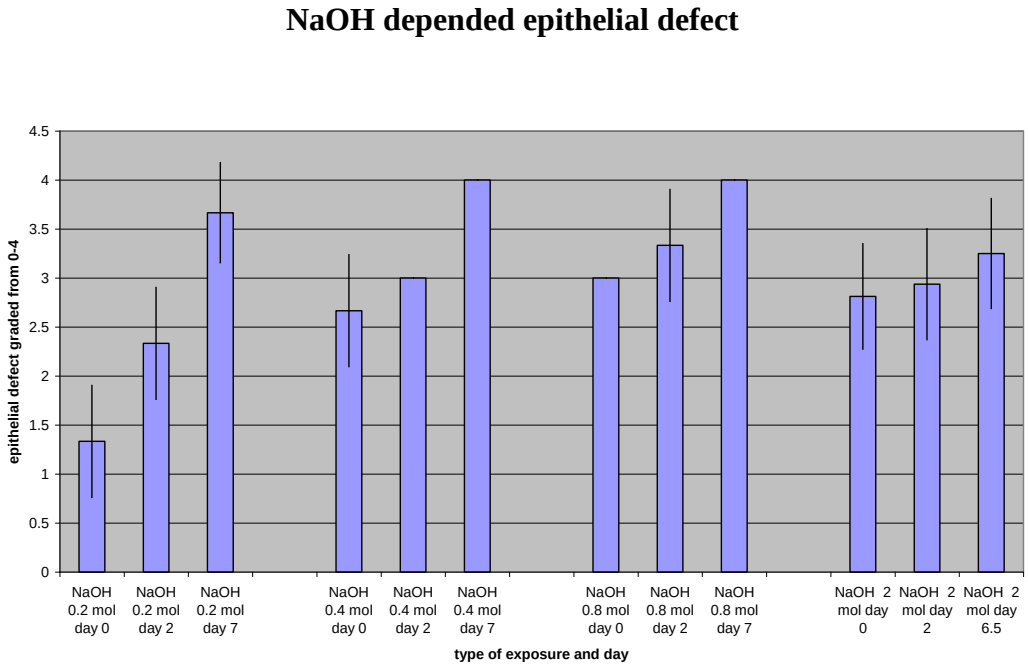


Fig. 11: Epithelial defects of ex vivo corneas at different time points after exposure to various concentrations of NaOH.

Abscissa: experimental groups/type of exposure (n=3 each); ordinate: score of epithelial defects (mean values ans standard deviations)

The size of the initial epithelial defects after burn with NaOH increased from 0.2 to 0.4 mol NaOH, suggesting dose responses. In the following groups, the experimental data were insufficient being only one case with 0.8 mol NaOH, and with 2.0 mol NaOH the application method was different, so that the resulting effects after burn might have been different. Two days after the exposure to NaOH, dose responses were remarkable between 0.2, 0.4 and 0.8 mol. On day seven, the epithelial defects of the ex vivo corneas were the same size, showing finally a maximum effect of the NaOH burn.

H_2O_2 dependent epithelial defect

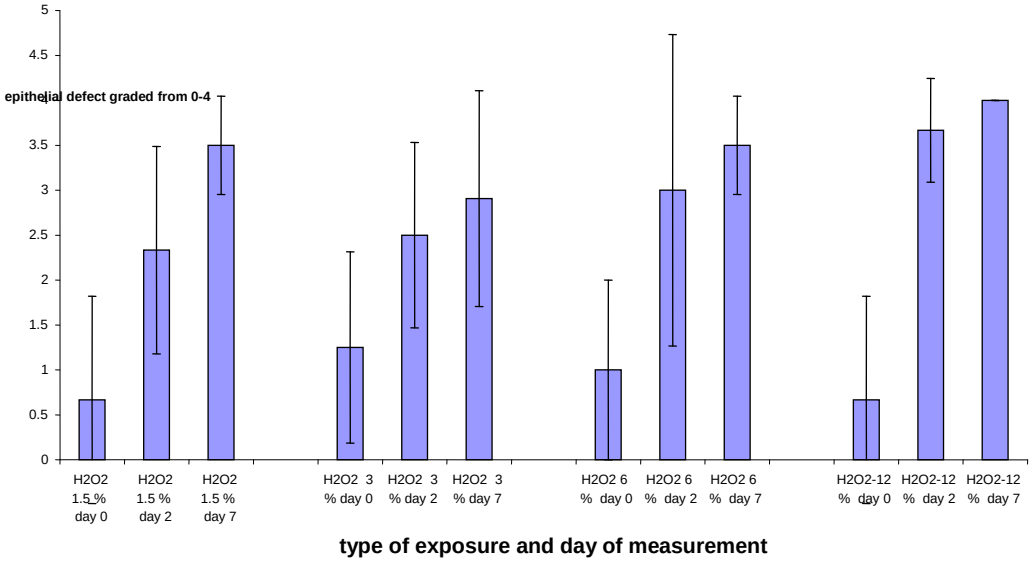


Fig. 12: Epithelial defects of ex vivo corneas at different time points after exposure to various concentrations of H_2O_2.
Abscissa: experimental groups/type of exposure (n=3 each); ordinate: score of epithelial defects (mean values ans standard deviations)

In the experiments with H_2O_2 no dose response could be seen when testing different concentrations of the substance. The initial defects after burn were very small but enlarged with time, reaching nearly equally high scores on day seven, showing that the damage due to the burn increased during the course of the experiment. With the higher concentrations (6% and 12%) the maximal defect could already be seen on day two.

TCA dependent epithelial defect

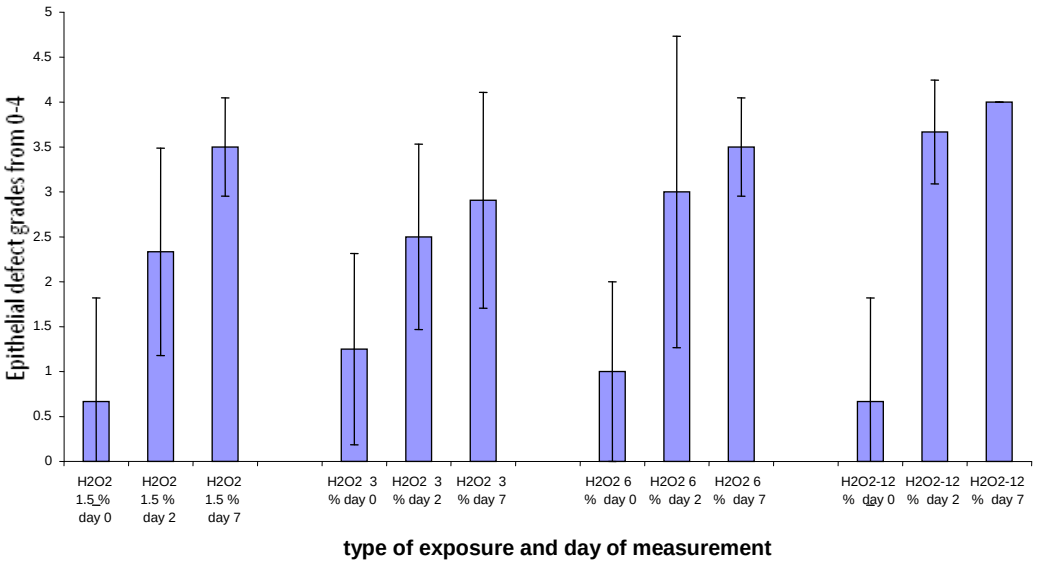


Fig. 13: Epithelial defects of ex vivo corneas at different time points after exposure to various concentrations of TCA.

Abscissa: experimental groups/type of exposure (n=3 each) ; ordinate: score of epithelial defects (mean values ans standard deviations)

Directly after TCA exposure, the common size of erosion was close to the size of the contact lens applicator. The average damaged zone increased with time, but the large standard deviations showed that the defects of the epithelium were less stable, especially, when the lower concentrations of TCA were used, indicating here healing tendencies. The application of 30% TCA shows already on day 2 maximum damage and no healing at all.

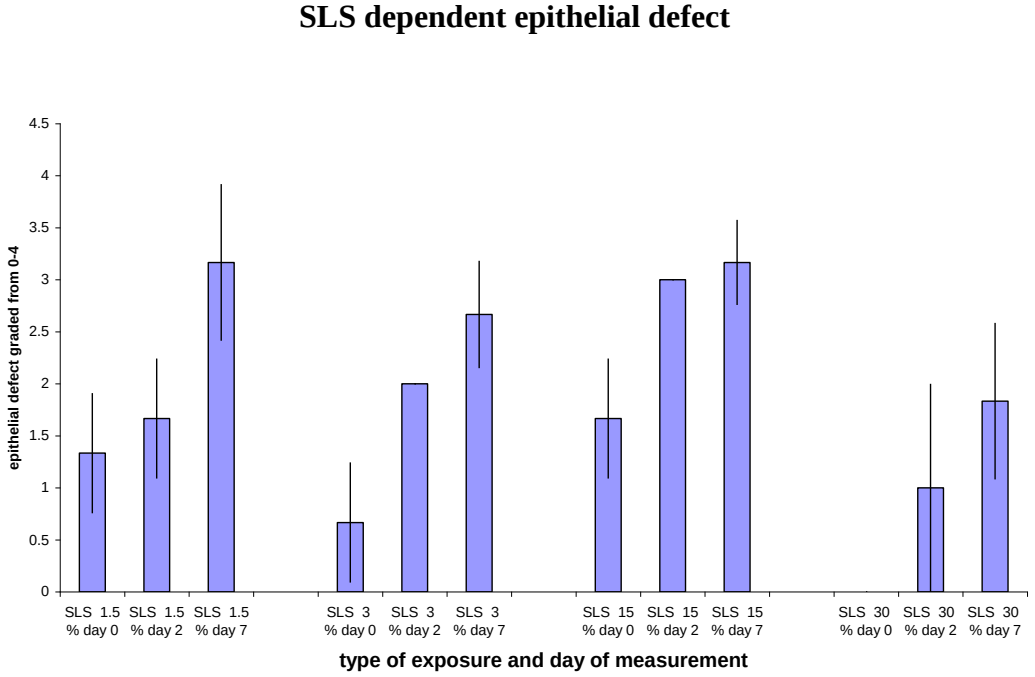


Fig. 14: Epithelial defects of ex vivo corneas at different time points after exposure to various concentrations of SLS.

Abscissa: experimental groups/type of exposure (n=3 each; ordinate: score of epithelial defects (mean values ans standard deviations)

After SLS exposure, the epithelial defects increased with time, reaching almost equal maximum values after 7 days. With lower concentrations of SLS the damage to the epithelium developed more slowly. After application of 30 % SLS, the corneal damage seemed to be less than with lower concentrations, and showed clinically some healing in the central zone of the ex vivo cornea.

Synopsis of epithelial defects

Comparing the effects of different chemicals applied to the ex vivo corneas, with all substances tested, the size of epithelial lesions increased during the time course of the experiments, when lower concentrations were used. After exposure to higher concentrations of the chemicals selected, mean values and very high standard deviations of epithelial scores suggested, that epithelial lesions were near maximum damage already on day two, so that no further progression in damage could be observed during the following days. Increased scores of epithelial defects showing dose responses were found on the day of burn (day 0) between 0.2 and 0.4 mol NaOH, and from 1.5% to 3.0% H_2O_2. On day two, significantly increased scores of epithelial lesions were consistent

with dose responses only with NaOH from 0.2 to 0.8 mol. With the other chemicals, where mean values also may suggest dose responses, the standard deviations were so large, that these results could not be accepted as such.

Endothelial damage

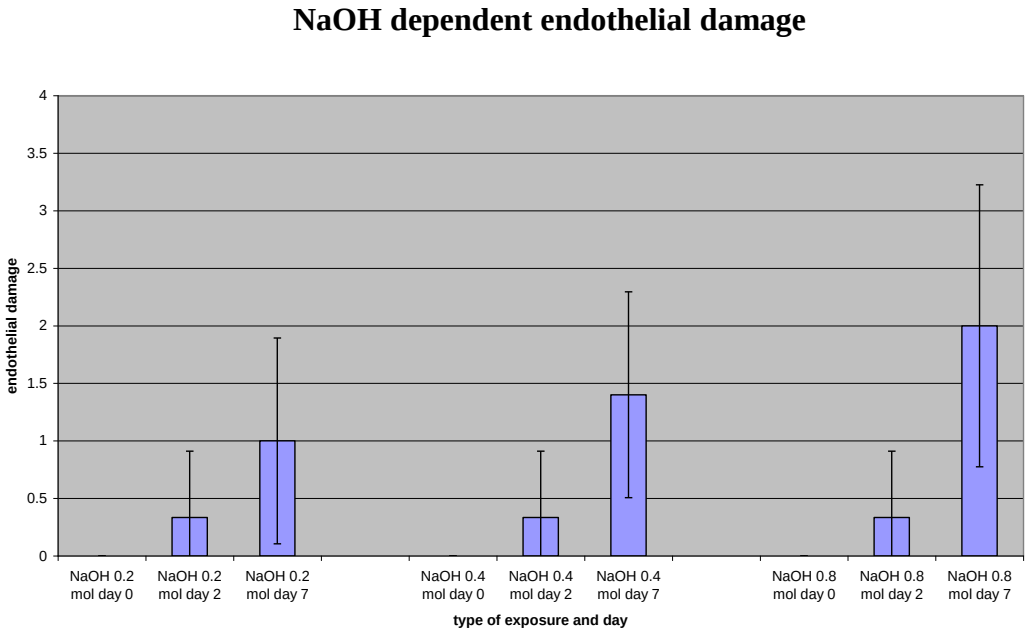


Fig. 15: Damage to the endothelium of ex vivo corneas after exposure of the epithelial surface to 0.2, 0.4, and 0.8 mol NaOH.

Abscissa: experimental groups/type of exposure; ordinate: score of endothelial defects (mean values and standard deviations)

At the time when the experiments with exposure to 2.0 mol NaOH were performed, endothelial photography was not yet established in the system, so that for this substance only data for the concentrations of 0.2, 0.4 and 0.8 are available. An increasing damage of endothelial cells was observed 7 days after exposure to NaOH. With the lower concentrations, only single cells were affected, while with 0.8 mol NaOH a higher amount of necrosic stripes were detected. The endothelial damage on day 7 showed a clear dose response.

H_2O_2 dependent endothelial damage

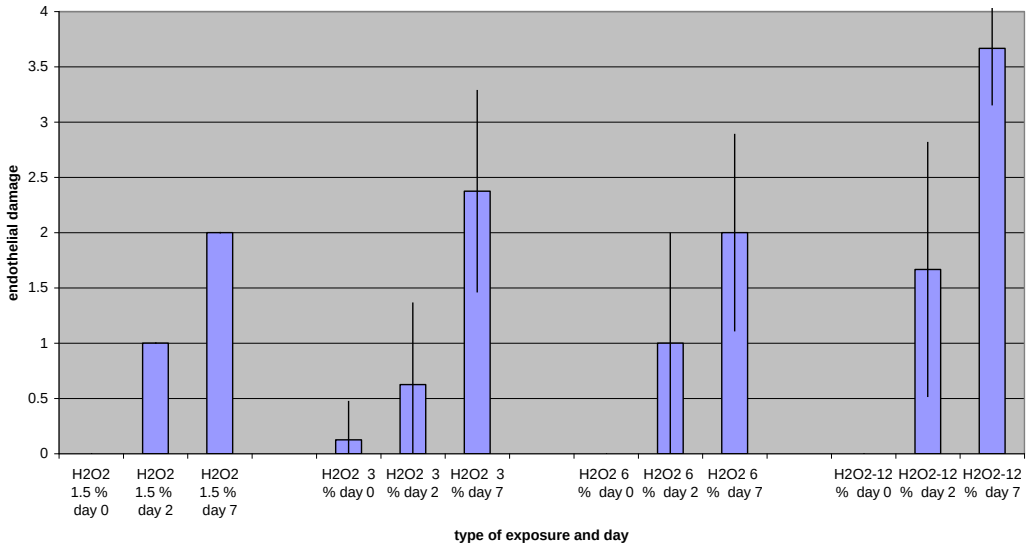


Fig. 16: Damage to the endothelium of ex vivo corneas after exposure of the epithelial surface to 1.5%; 3.0%; 6.0% and 12.0% H2O2.

Abscissa: experimental groups/type of exposure; ordinate: score of endothelial defects (mean values and standard deviations)

Immediately after exposure to H_2O_2, the endothelium of the ex vivo corneas did not yet exhibit any damage visible with the specular microscope. But on day 2 and 7 increasing alteration of the endothelium was observed. Endothelial damage on day 2 shows clear dose responses, which may become statistically significant with a larger number of cases. Seven days after exposure to 12% H_2O_2, the damage to the endothelium seemed at maximum, with highest scores and almost total endothelial necrosis visible. In the experiments with 3% and 6% H_2O_2, the standard deviations on day 7 were rather large suggesting also very severe, if not maximum damage to the endothelium.

TCA dependent endothelial damage

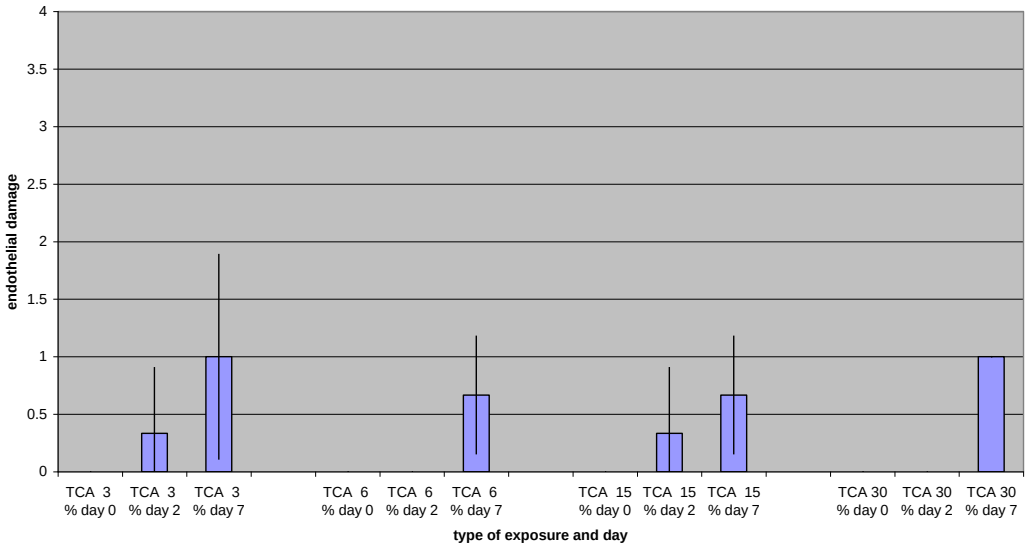


Fig. 17: Damage to the endothelium of ex vivo corneas after exposure of the epithelial surface to 3.0%; 6.0%; 15.0% and 30.0% TCA.
Abscissa: experimental groups/type of exposure; ordinate: score of endothelial defects (mean values and standard deviations)

The damage observed after exposure to TCA was not very prominent and not dose related. Single endothelial cells were stained with trypan blue. This is consistent with the observation of a sharply limited damage in the stromal tissue, without further diffusion into deeper layers of the cornea. So, only little endothelial damage was achieved by exposure to TCA, but the little numbers of experiments did not allow for more evaluation.

SLS dependent endothelial damage

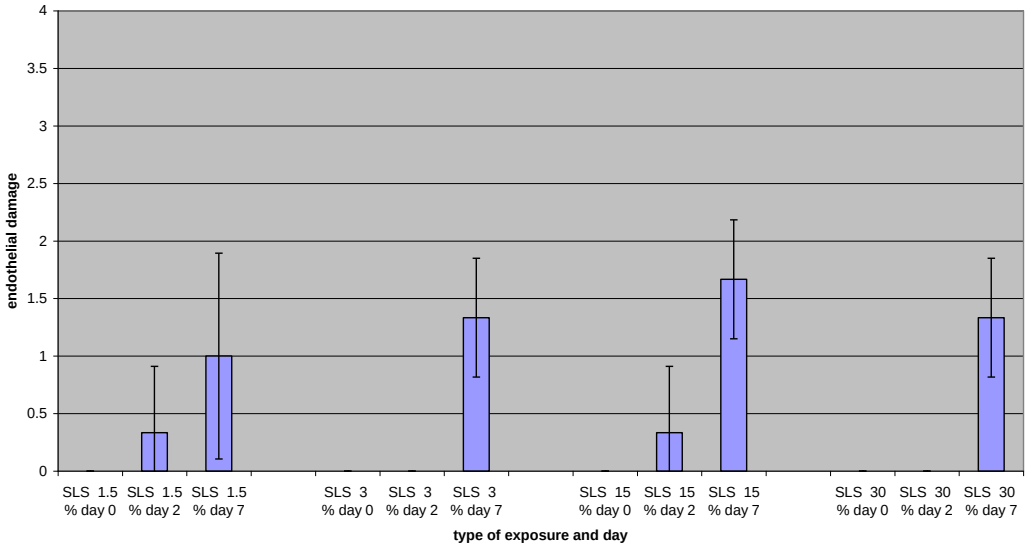


Fig. 18: Damage to the endothelium of ex vivo corneas after exposure of the epithelial surface to 1.5%; 3.0%; 15.0% and 30.0% SLS.

Abscissa: experimental groups/type of exposure; ordinate: score of endothelial defects (mean values and standard deviations)

SLS does not show severe endothelial damage. It seems to exist concentration dependent increasing lesions of low degree with the concentrations of 1,5%, 3% and 15% at day 7.The group of corneas treated with 30% SLS however showed much lower effects.

pH Measurement and Glucose-/Lactate monitoring

pH in the perfusion medium

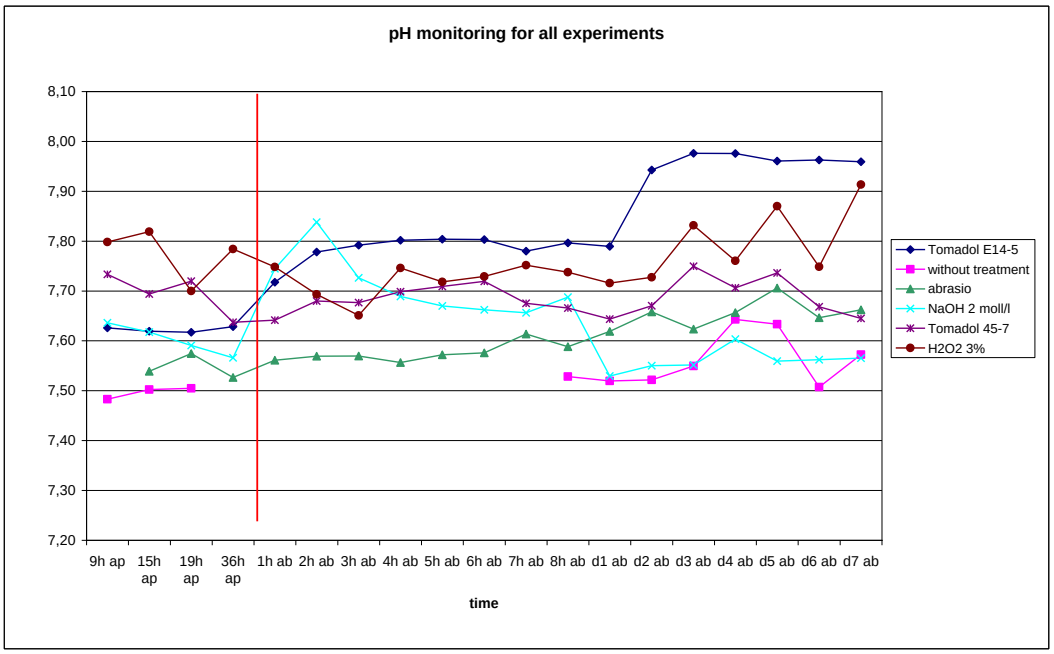


Fig. 19: pH in the perfusion medium over time. Means +/- STD in mmol/l of all groups exposed to chemicals.

Ex vivo corneas were pre-cultured for 48 hours. The vertical line marks the time of exposure. The pH values were measured after exposure for 8 hours every hour, then once every day up to 7 days. Abscissa: time scale; ordinate: pH; the experimental exposure is explained on the right side of the diagram. Each point refers to n=16 single ex vivo corneas.

As described above loss of carbondioxide through the PVC-tubings was a severe problem at the beginning of the testings. After having installed the CO_2- supply to guarantee a constant carbondioxide atmosphere gas exchange system, it was possible to stabilise the pH of the inflowing MEM-medium, so that we achieved nearly physiological pH values in the perfusion medium.

In this first series of experiments, the pH values varied between 7.5 and 7.8. During the incubation period of the 48 hours after mounting the ex vivo cornea in the perfusion chamber, the pH values converged to values between 7.5 and 7.7, except the corneas, which were later exposed to H_2O_2. After exposure, in the NaOH exposed group, the pH in the perfusion medium increased for two hours to 7.84. From day 2, in the ex vivo corneas exposed to tomadol E14-5 the pH increased to 7.98.

Glucose and lactate levels in the perfusion medium

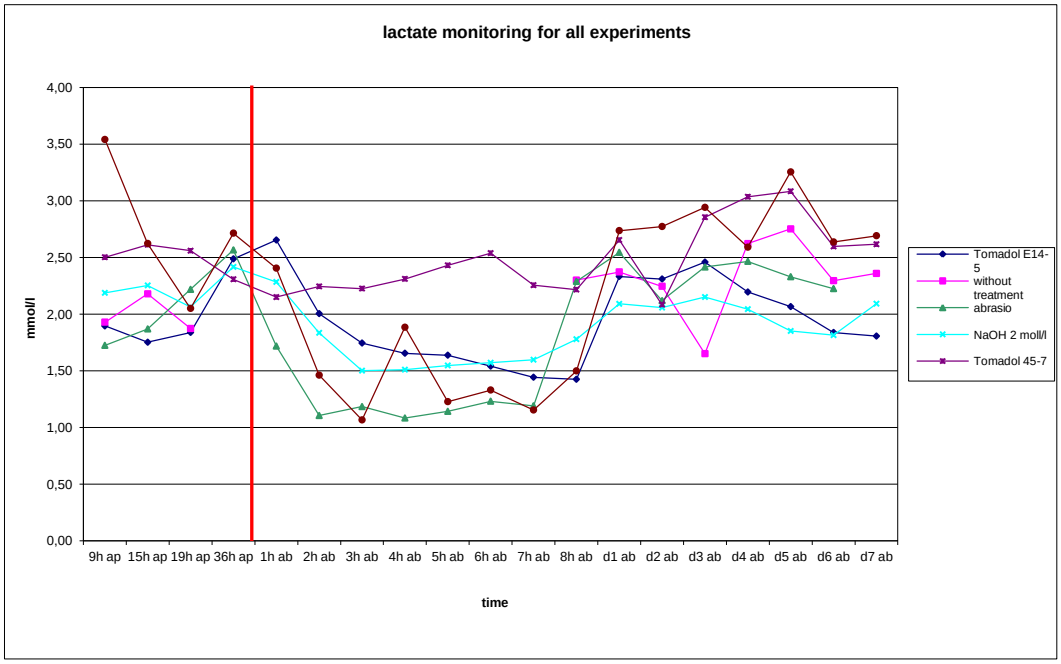


Fig. 20: Lactate levels in the perfusion medium over time. Means +/- STD in mmol/l of all groups exposed to chemicals.

Ex vivo corneas were pre-cultured for 48 hours. The vertical line marks the time of exposure. The lactate levels were measured after exposure for 8 hours every hour, then once every day up to 7 days. Abscissa: time scale; ordinate: lactate levels in mmol/l. The experimental exposure is explained on the right side of the diagram. Each point refers to n=16 single ex vivo corneas.

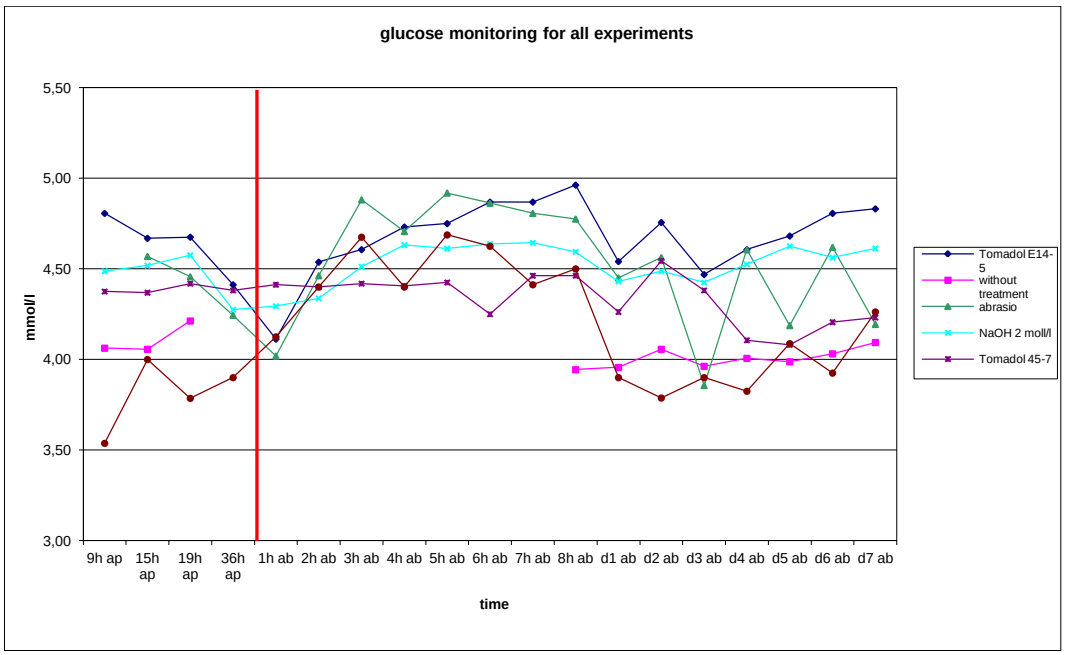


Fig. 21: Glucose levels in the perfusion medium over time. Means +/- STD in mmol/l of all groups exposed to chemicals.

Ex vivo corneas were pre-cultured for 48 hours. The vertical line marks the exposure. The glucose levels were measured after exposure for 8 hours every hour, then once every day up to 7 days. Abscissa: time scale; ordinate: glucose levels in mmol/l. The experimental exposure is explained on the right side of the diagram. Each point refers to n=16 single ex vivo corneas.

Continuous measurements of glucose, lactate and pH in the tubings behind the artificial anterior chamber and in the supernatants were performed. These analyses proved the vitality of the ex vivo cornea in the culturing system of the perfusion chamber. Among other nutrient metabolites, the perfusion medium contained about 5.56 mmol glucose, but no lactate. Glucose consumption of the ex vivo cornea and its lactate production could be measured in the perfusion medium by a decrease in the glucose levels and by an increase in lactate. A prominent decrease in lactate production of the ex vivo cornea, in pre-experimental culturing phase was a criterium to exclude the corneas from further experiments. The cut off level for this was defined below 1 mmol/lactate per litre MEM. Before the exposition, the lactate levels were inconstant and merged to about 2.5 mmol at the end of the preincubation period. After exposure to the toxic chemicals, lactate levels decreased to values between 1.0 and 1.5 mmol, except those of the corneas exposed to tomadol 45-7. After one day, lactate levels returned to values before exposure.

Like the lactate, the glucose levels varied considerably after preparation of the cornea from the globe and mounting in the perfusion chamber. But when the ex vivo corneas

were incubated for 48 hours in the perfusion system, glucose levels converged versus 4.0 to 4.5 mmol. After the exposure to the chemical test substances, glucose levels increased in varying extent for about one day, changing inversely to the lactate levels.

pH, glucose and lactate in untreated corneas

Perfusion medium

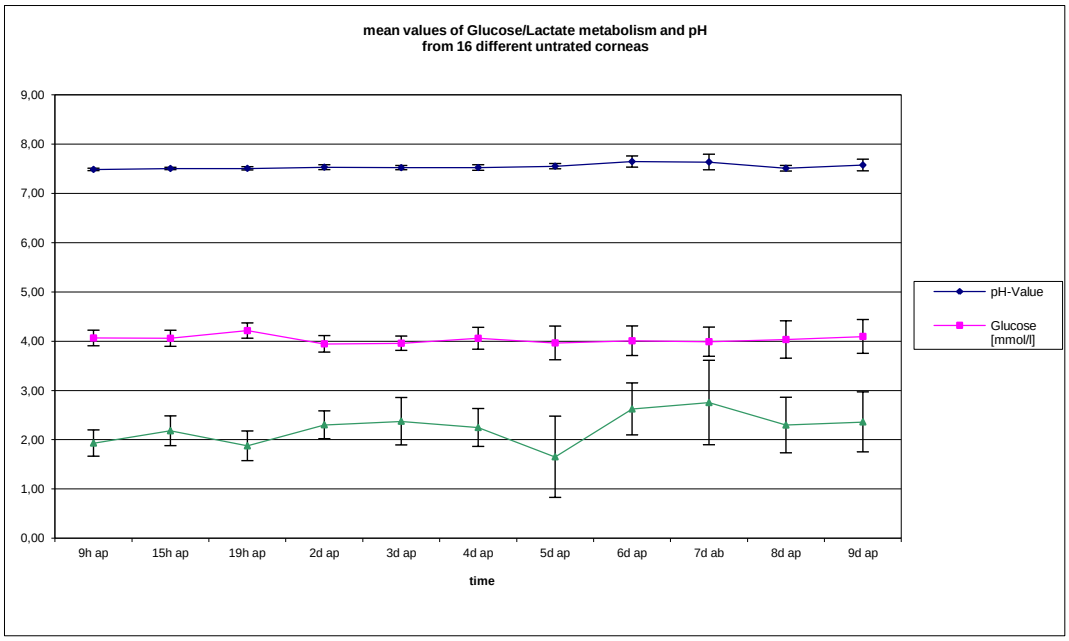


Fig. 22: Analyses of the perfusion medium from unexposed, healthy ex vivo corneas over 9 days. Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16).

In the testings of untreated corneas, i.e. corneas, that were not exposed to any substance, the pH in the perfusion medium remained constantly on 7.5. The glucose concentration in the inflowing culture medium was set on 5.56 mmol. In the perfusion medium at the outflow of the perfusion chamber, a level of 4 mmol glucose equilibrated constantly by uptake into the corneal metabolism. Lactate was not contained in the inflowing culture medium, but could be measured in the outflowing medium. The lactate level lightly increased during the 9 days of cultivation from about 2mmol at the begin of the tests to about 2.3-2.5mmol in the follwing days. It should be noted, that the standard deviations were rather small for pH and glucose levels, and for lactate still acceptable.

Supernatant rinsing medium

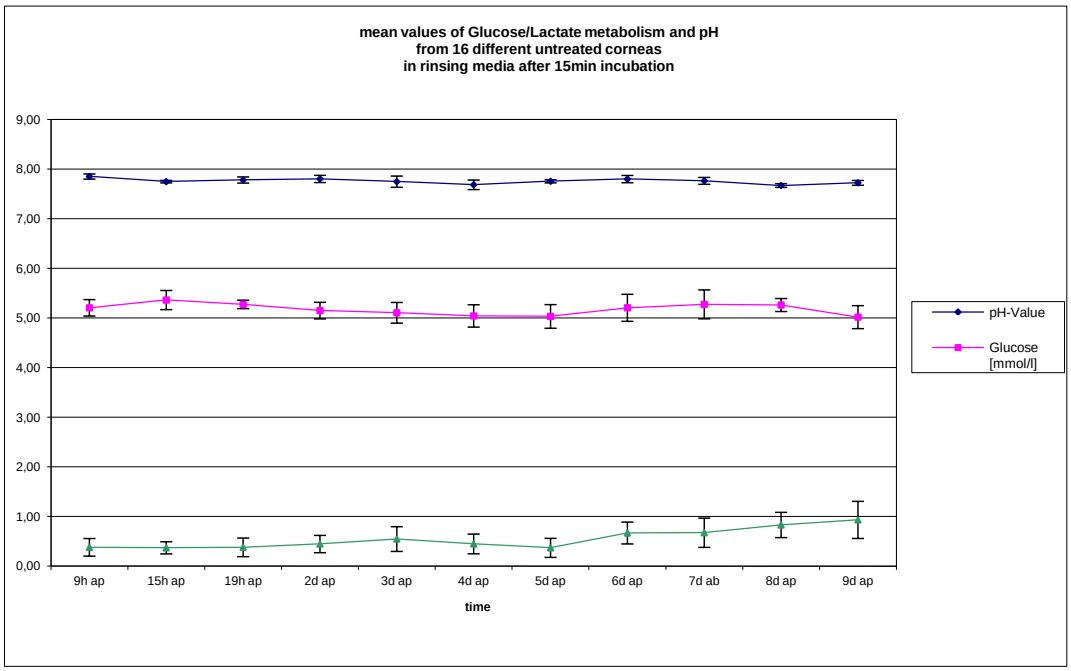


Fig. 23: Analyses of the supernatant medium rinsed for 15 min for sampling of metabolites on the surface of unexposed healthy ex vivo corneas over 9 days.
The ordinate gives pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16).

The pH on the corneal epithelial surface remained constantly on 7.8, slightly more alkaline than in the perfusion chamber. The glucose concentration of the culture medium was set on 5.56 mmol. On the corneal suface, levels of about 5.2 mmol glucose were found. Lactate is not contained in the culture medium. Low concentrations of less than 1mmol/l of lactate were measured in the supernatant rinsing medium after flushing the corneas.

pH, glucose and lactate in abraded corneas

Perfusion medium

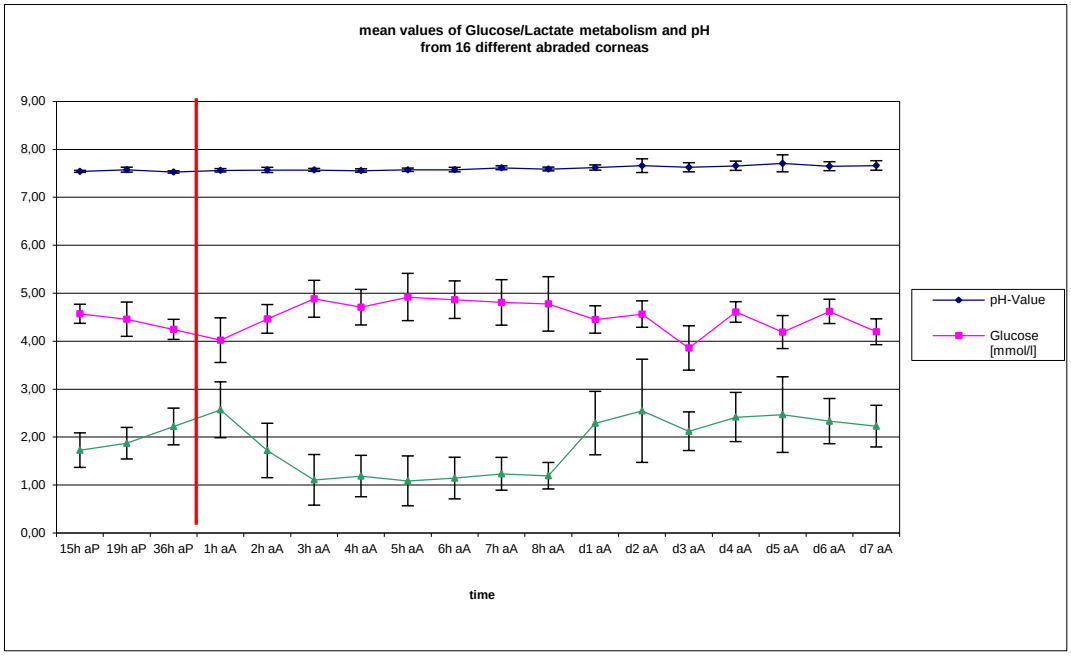


Fig. 24: Analyses of the perfusion medium from ex vivo corneas over 9 days, in which, after an incubation period of 48 hours, the epithelium was removed mechanically.
Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the abrasio of the epithelium

The pH remained stable at 7.5 during the whole time of the experiment. After the abrasion in the medium, that passed through the perfusion chamber, the glucose levels were increased and corresponding lactate levels were decreased by about one mmol each for 8 hours. On the next analyses, one day after the abrasion of the epithelium, glucose and lactate levels returned to the initial levels and so remained until to the seventh day after abrasion.

Supernatant medium

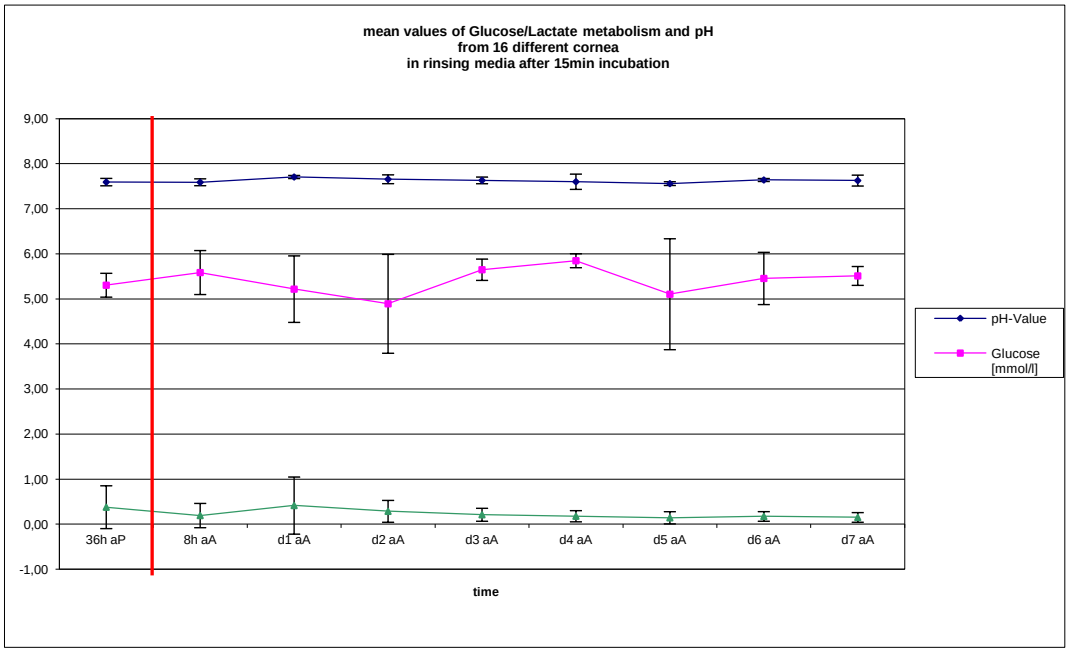


Fig. 25 Analyses of the supernatant medium rinsed for 15 min for sampling of metabolites on the surface of ex vivo corneas over 9 days, in which, after an incubation time of 48 hours, the epithelium was removed mechanically.

Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the abrasio of the epithelium

On the corneal surface, pH remained constantly at about 7.5. The glucose levels, with some variation, remained constantly on the level given by the rinsing medium. The lactate levels were rather low over the whole time of the experiment. As no lactate was contained in the rinsing medium, only very little lactate was released from the surface.

pH, gucose and lactate in Tomadol E14-5 10% treated corneas

Perfusion medium

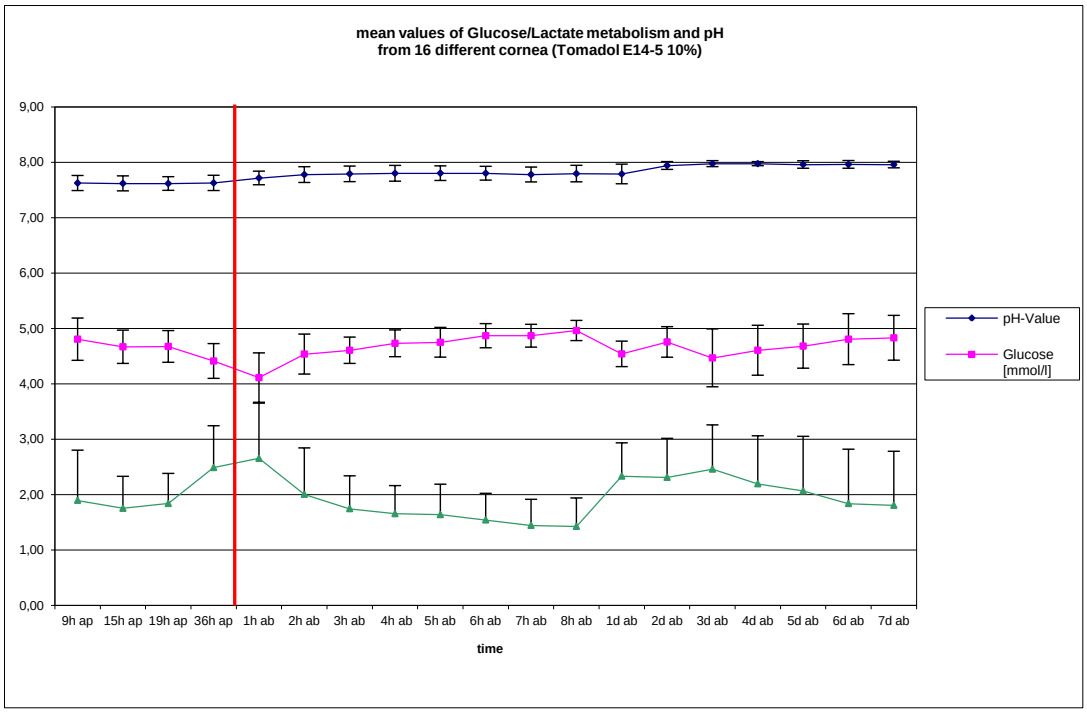


Fig. 26: Analyses of the perfusion medium from ex vivo corneas over 9 days, in which, after an incubation period of 48 hours, 10 % tomadol E 14-5 was applied to the corneal surface.
Abscissa: time scale; ordinate:pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the exposure of the ex vivo corneas to tomadol E 14-5.

The pH remained stable at 7.5 during the whole time of the experiment. After the exposure of the ex vivo corneas to 10 % tomadol E14-5 in the medium, that passed through the perfusion chamber, the glucose levels were increased and corresponding lactate levels were decreased by about one mmol each for 8 hours, On the next analyses, day one after the exposure, glucose and lactate levels returned to their initial levels, but so remained only for 3 days. Then glucose and lactate levels diverged again.

Supernatant medium

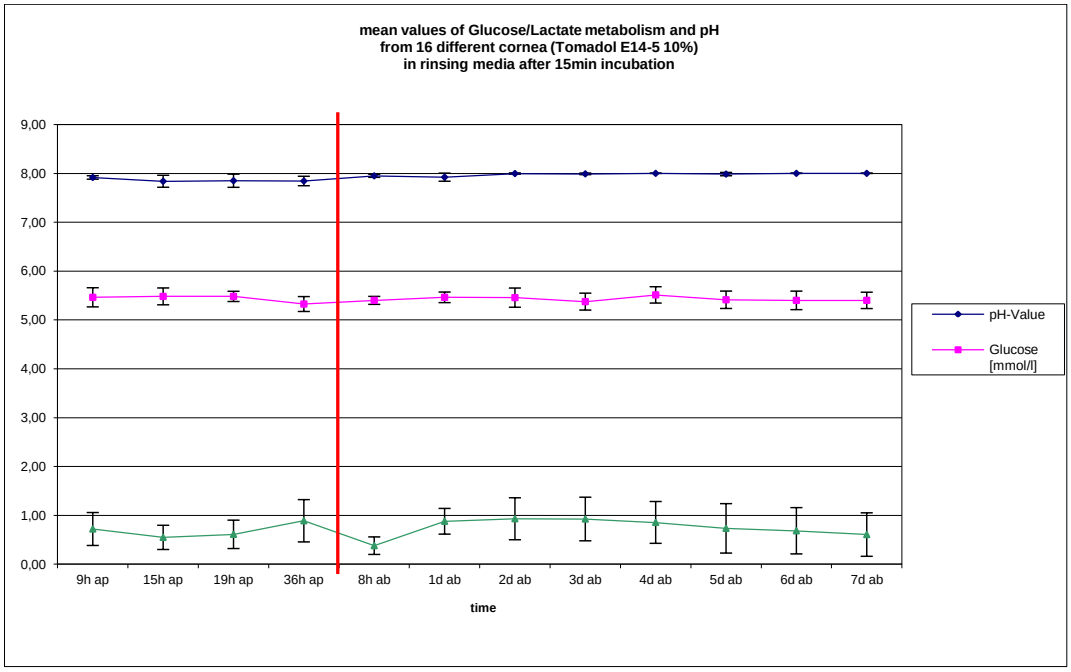


Fig. 27: Analyses of the supernatant medium rinsed for 15 min for sampling of metabolites on the surface of ex vivo corneas over 9 days, in which, after an incubation period of 48 hours, 10 % tomadol E 14-5 was applied to the corneal surface.
Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the exposure of the ex vivo corneas to tomadol E 14-5.

On the corneal surface, pH rose to 8.0. The glucose, with some variation, remained constantly on the level given by the rinsing medium. As no lactate was contained in the rinsing medium, only very little lactate was released from the epithelium to the surface, which shows very low glucose/lactate metabolism.

pH, glucose and lactate in Tomadol 45-7 3% treated corneas

Perfusion medium

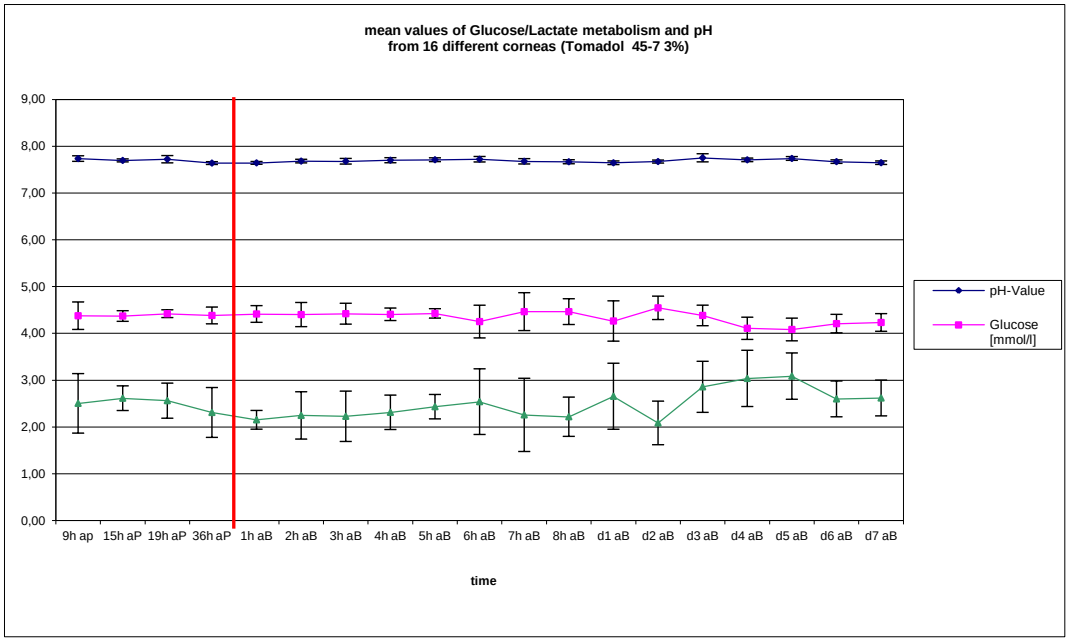


Fig. 28: Analyses of the perfusion medium from ex vivo corneas over 9 days, in which, after an incubation period of 48 hours, 3 % tomadol 45-7 was applied to the corneal surface.
Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the exposure of the ex vivo corneas to 3 % tomadol 45-7.

After the exposure of the ex vivo corneas to 3 % tomadol 45-7 in the medium, that had passed through the perfusion chamber, pH remained constantly at 7,8. The glucose levels were found to be at 4,5 mmol, being lower by one mmol than the given concentration of the inflowing culture medium. The lactate level was found between 2 and 3 mmol. There was virtually no change of the glucose and lactate levels for about 7 hours after exposure to tomadol 45-7. Then their mean values varied and their standard deviations increased, both, glucose and lactate, presented some divergent variation, but remained in the average on the levels indicated. In the late phase of the experiments, beginning 3 days after the exposure, glucose levels decreased and lactate increased slightly.

Supernatant medium

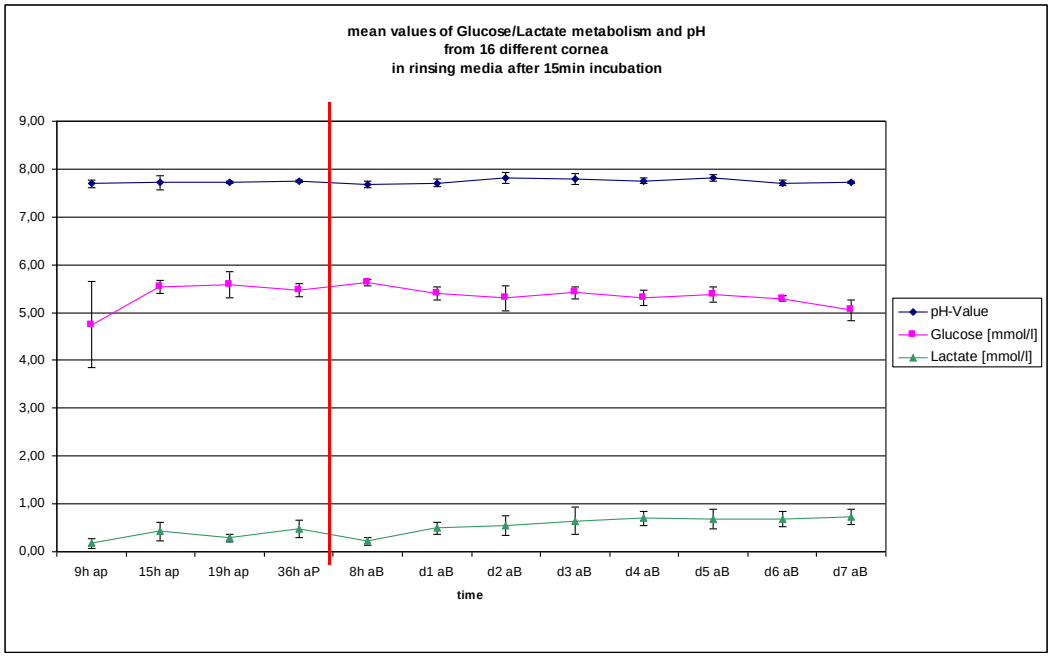


Fig. 29: Analyses of the supernatant medium rinsed for 15 min for sampling of metabolites on the surface of ex vivo corneas over 9 days, in which, after an incubation period of 48 hours, 3 % tomadol 45-7 was applied to the corneal surface.

Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the exposure of the ex vivo corneas to tomadol 45-7.

On the corneal surface, pH remained constantly at 7.8. The glucose, with some variation, remained constantly on the level given by the rinsing medium. As no lactate was contained in the rinsing medium, only very little lactate was released to the surface, which shows very low glucose/lactate metabolism.

pH, glucose and lactate in H_2O_2 treated corneas

Perfusion medium

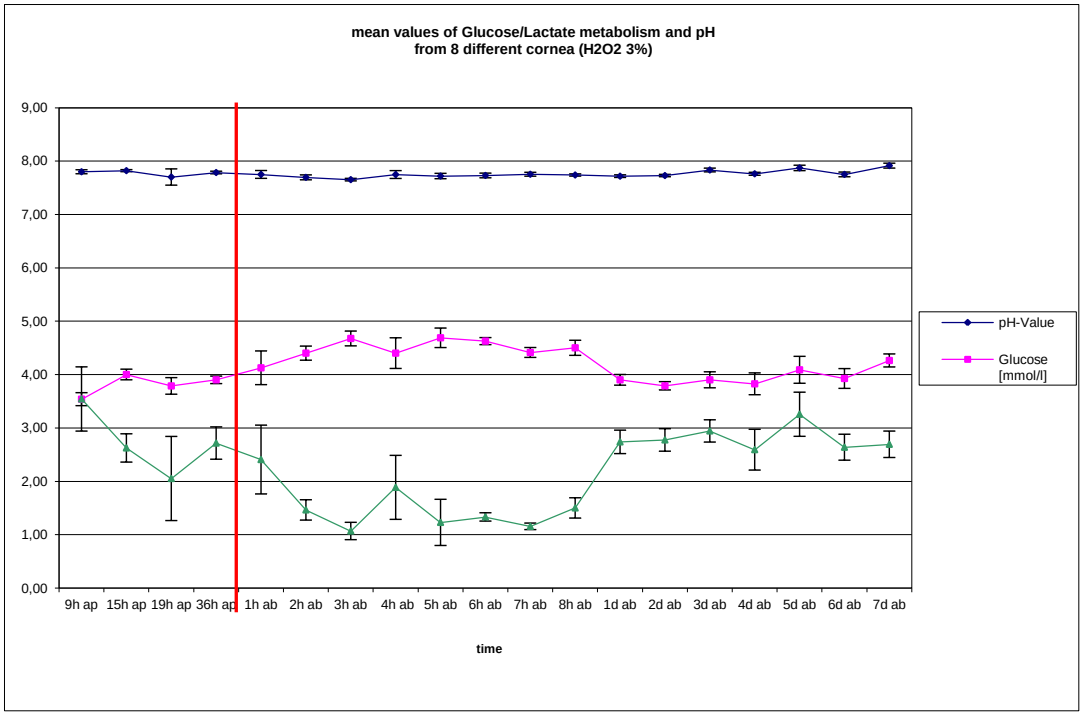


Fig. 30: Analyses of the perfusion medium from ex vivo corneas over 9 days, in which, after an incubation period of 48 hours, 3% H2O2 was applied to the corneal surface.
Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the exposure of the ex vivo corneas to 3% H2O2.

The pH remained stable at 7.8 during the whole time of the experiment. After the exposure of the ex vivo corneas to 3% H_2O_2 in the medium, that passed through the perfusion chamber, the glucose levels increased from 4,0 to 4,8 mmol. At the same time, lactate levels decreased by about 2 mmol for 8 hours. On the next analyses, one day after the exposure to 3% H_2O_2, glucose and lactate had returned to their initial levels, and so remained until to the end of the experiment after 7 days

Supernatant medium

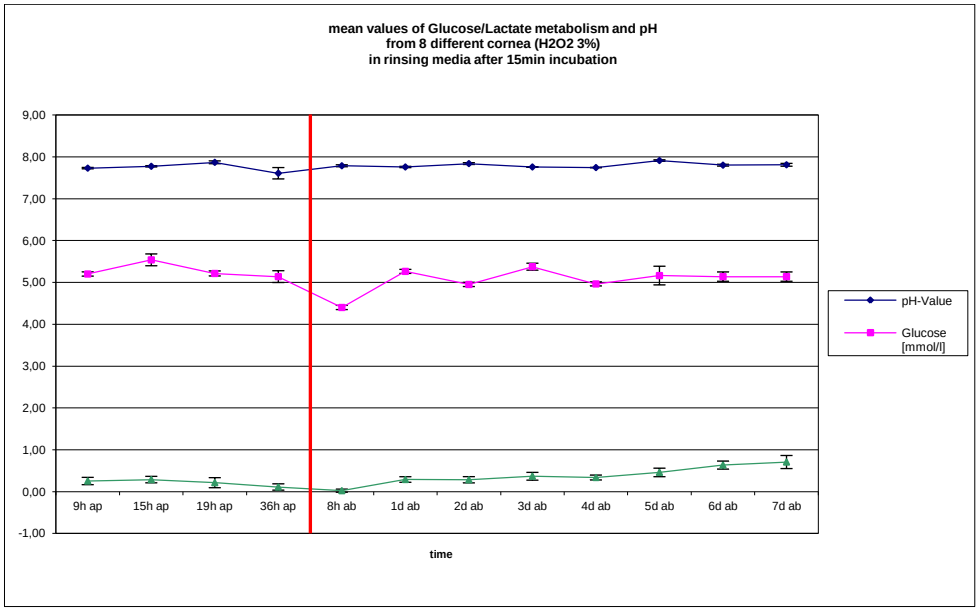


Fig. 31: Analyses of the supernatant medium rinsed for 15 min for sampling of metabolites on the surface of ex vivo corneas over 9 days, in which, after an incubation period of 48 hours, 3% H2O2 was applied to the corneal surface.
Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=8). The vertical line at 48 hours marks the exposure of the ex vivo corneas to 3% H2O2.

On the corneal surface pH remained constantly at 7.8. The glucose, with some variation, remained constantly on the levels given by the rinsing medium. As no lactate was contained in the rinsing medium, only very little lactate was released to the surface, which shows only low glucose/lactate metabolism.

pH, glucose and lactate in NaOH 2 mol/l treated corneas

Perfusion medium

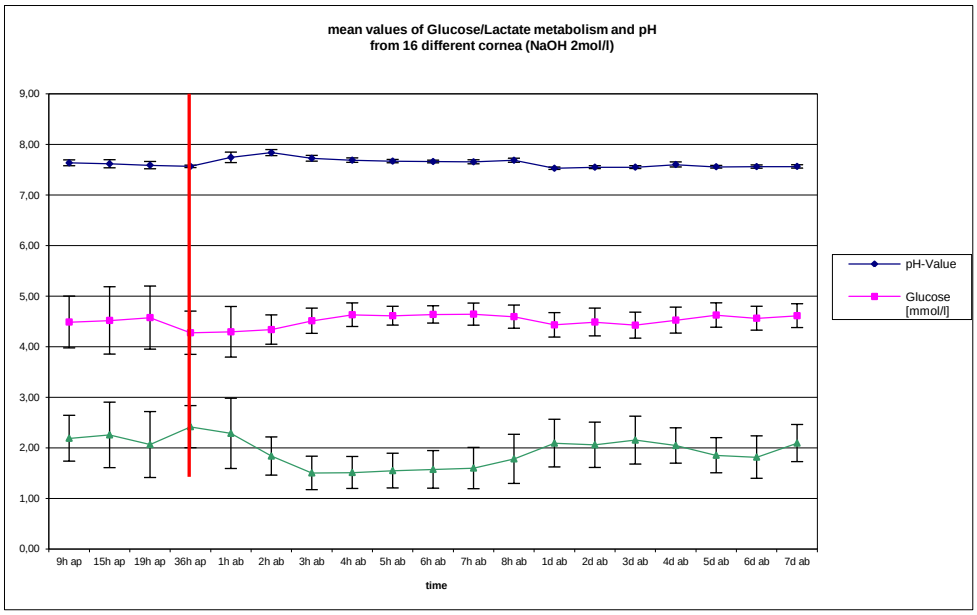


Fig. 32: Analyses of the perfusion medium from ex vivo corneas over 9 days, in which, after an incubation period of 48 hours, 2 mol NaOH was applied to the corneal surface.
Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the exposure of the ex vivo corneas to 2 mol NaOH.

The pH remained stable at about 7.8 during the whole time of the experiment. After the exposure of the ex vivo corneas to NaOH 2mol in the medium, that passed through the perfusion chamber, the glucose levels increased after 3hours from 4,2 to 4,8 mmol. At the same time, lactate levels decreased by about 1 mmol for 8 hours. On the following day glucose and lactate values normalized to the initial levels and so remained until the end of the experiment on day 7, parallel to the healing of the epithelium as demonstrated in fig 10.

Supernatant medium

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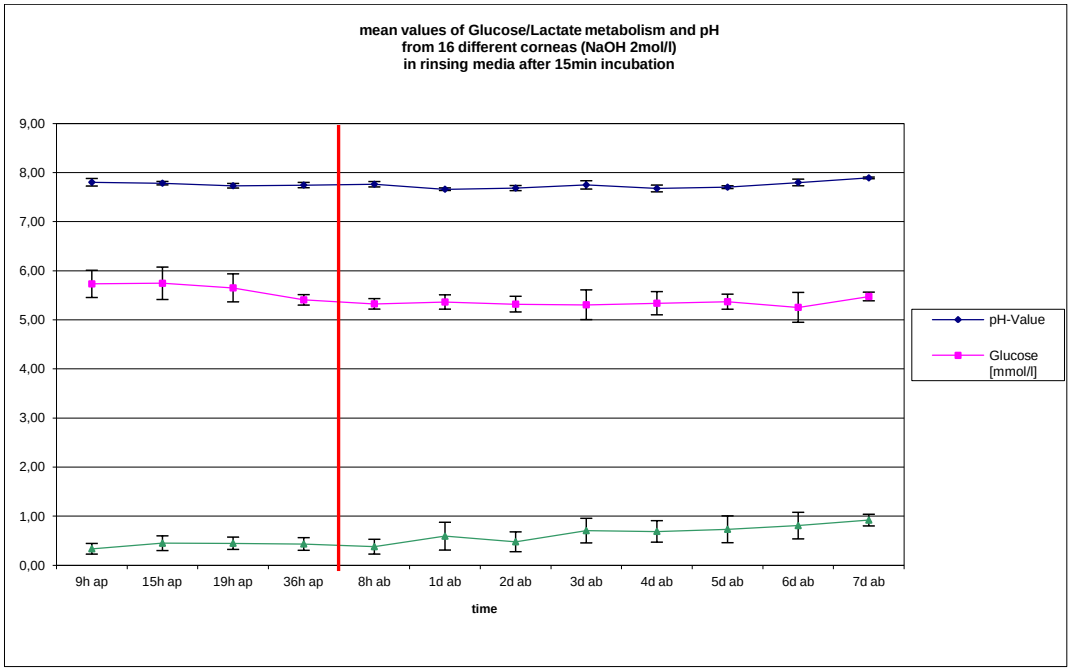

Fig. 33: Analyses of the supernatant medium rinsed for 15 min for sampling of metabolites on the surface of ex vivo corneas over 7 days, in which, after an incubation period of 48 hours, 2 mol NaOH was applied to the corneal surface.

Abscissa: time scale; ordinate: pH values and concentrations of glucose and lactate in mmol. Each symbol represents means +/- STD (n=16). The vertical line at 48 hours marks the exposure of the ex vivo corneas to 2 mol NaOH.

On the corneal surface, pH remained constantly at 7.8. The glucose, with some variation, remained constantly on the levels given by the rinsing medium. As no lactate was contained in the rinsing medium, only very little lactate was released to the surface, showing low metabolism

Dose response experiments in glucose/lactate metabolism

As described in the previous chapter, chemical injuries to the ex vivo corneas induced distinct responses of the glucose and lactate levels in the perfusion medium. After chemical damages to the cornea, the glucose levels in the perfusion medium increased, while the lactate levels decreased. This change of metabolite levels represented a disturbance of the glucose metabolism – the glycolysis - in the ex vivo cornea. In this chapter, various dosages of chemical agents were applied to investigate, whether dose responses may be obtained, when ex vivo corneas were exposed to different concentrations of the same chemical substances used before.

Dose response glucose/lactate metabolism in NaOH treated corneas

Perfusion medium

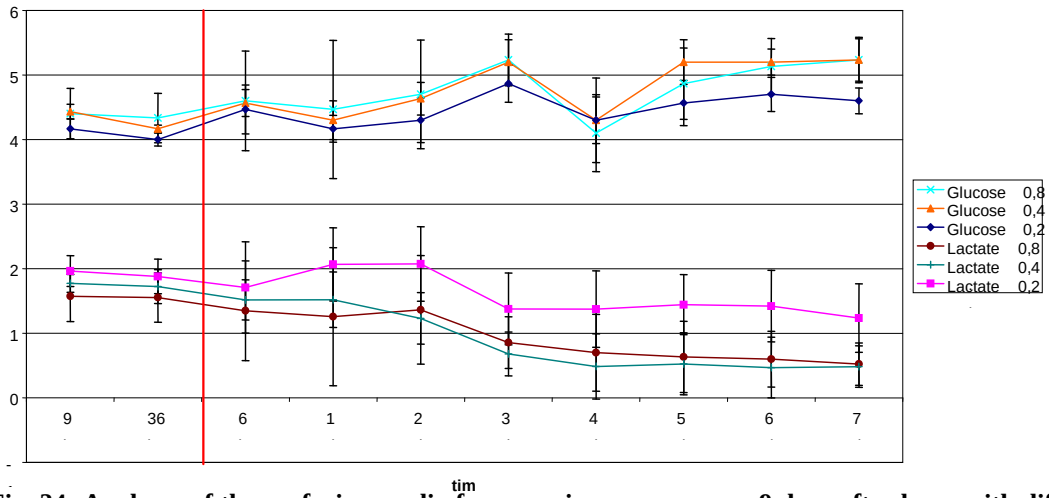

Fig. 34: Analyses of the perfusion media from ex vivo corneas over 9 days after burn with different concentrations of NaOH.

Abscissa: time scale [hours/days after burn]; ordinate: concentrations of glucose and lactate [mmol/l]. Each symbol stands for means +/- STD (n=3), the vertical line at 48 hours marks the time point of exposure of the ex vivo corneas to NaOH.

After the exposure of the corneal surface to 0.2, 0.4, and 0.8 mol NaOH, the expected increase of the glucose levels in the perfusion medium developed slowly, beginning on day 3. In the same time, also the lactate level responded to the chemical injuries and decreased. The changes of the glucose and lactate levels were less pronounced, when

mild alkali, 0,2 mol NaOH, was applied, while the exposure of the ex vivo cornea to 0.4 and 0.8 mol NaOH resulted in more prominent responses of the metabolite levels, however quite close to each other. It is worth to mention, that both, the increase of glucose, and the decrease of lactate lasted for longer than in the experiments reported in the previous chapter, where only 2 mol NaOH was used. At least for the lactate levels from days 5 to 7, the differences between the corneas exposed to 0.2 mol NaOH on the one side, and the burns with 0.4 and 0.8 mol NaOH on the other side, seemed to be statistically significant different, even in the small number of cases evaluated here.

Supernatant medium

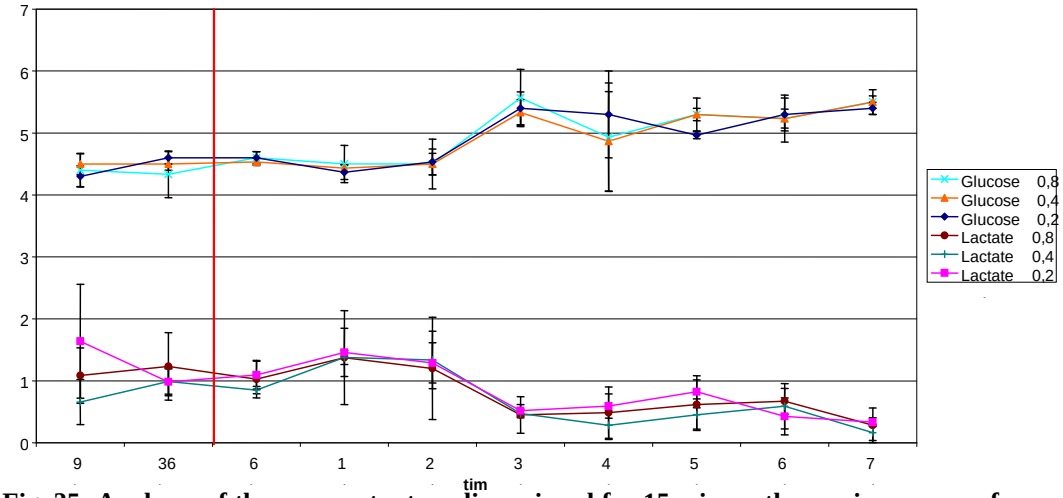

Fig. 35: Analyses of the supernatant medium rinsed for 15 min on the ex vivo corneas for sampling metabolites on the surface over 9 days after burn with different concentrations of NaOH.
Abscissa: time scale[hours/days after burn]; ordinate: concentrations of glucose and lactate [mmol/l]. Each symbol stands for means +/- STD (n=3), the vertical line at 48 hours marks the time point of exposure of the ex vivo corneas to NaOH.

In the same experiments, with all of the NaOH concentrations used for irritation of the ex vivo cornea, the supernatant rinsing media exhibited equal changes of the glucose and lactate levels in parallel to the results of the perfusion medium as presented in. However, the curves were almost identical for all concentrations of alkali used, .i e. there were no differences in the glucose and lactate levels found under different exposures. But the time course of the concentration changes, i.e. the increase of glucose and the decrease of lactate on day three, were almost identical with the perfusion medium.

Dose response glucose/lactate metabolism in H_2O_2 treated corneas

Perfusion medium

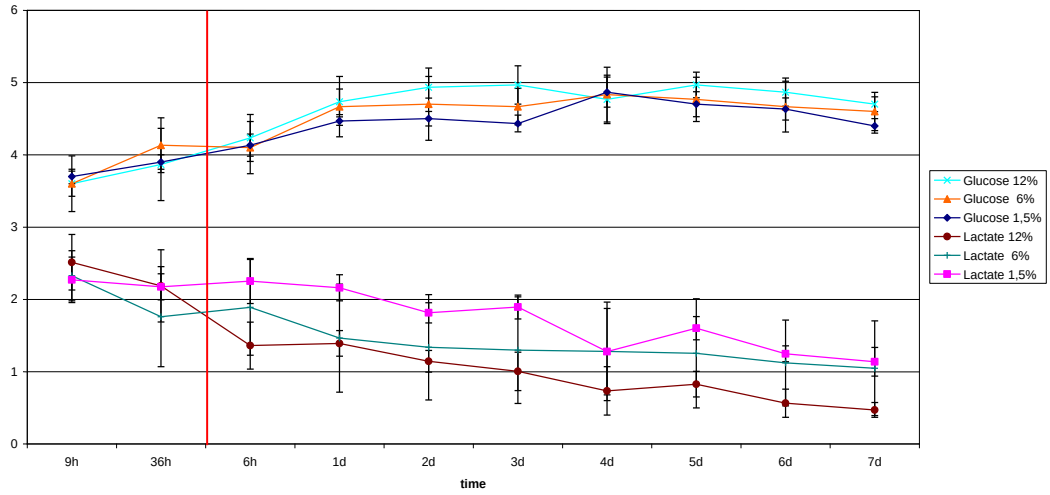

Fig. 36: Analyses of the perfusion media from ex vivo corneas over 9 days after burn with different concentrations of H2O2 .

Abscissa: time scale [hours/days after burn]; ordinate: concentrations of glucose and lactate [mmol/l]. Each symbol stands for means +/- STD (n=3), the vertical line at 48 hours marks the time point of exposure of the ex vivo corneas to H2O2.

In the perfusion medium of the ex vivo corneas exposed to various concentrations of H_2O_2, as after NaOH exposure, the glucose levels increased and the lactate levels decreased. Also, the changes in the levels of the two metabolites were larger with the higher concentration of the irritant. But different from the irritation by NaOH, after exposure to H_2O_2, the changes of the glucose and lactate levels began immediately after the exposure.

Supernatant medium

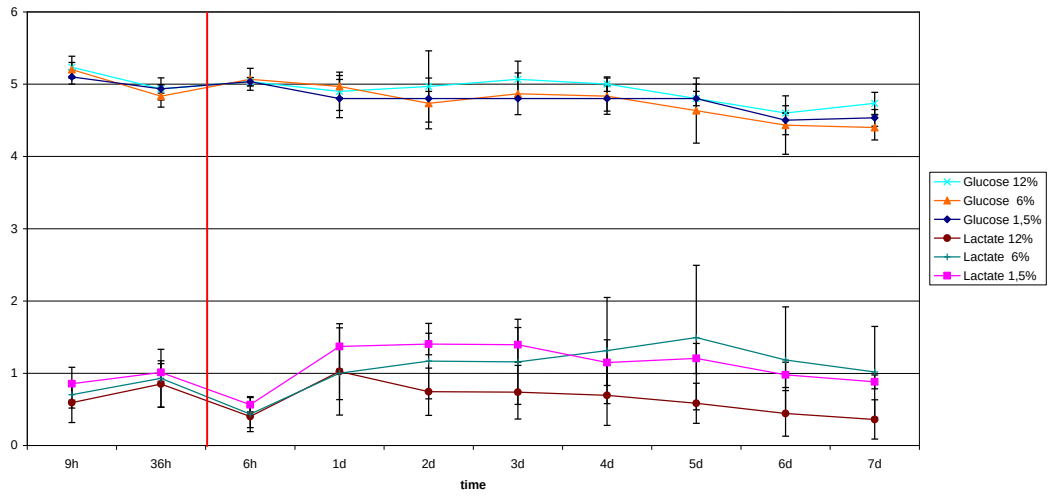

Fig. 37: Analyses of the supernatant medium rinsed for 15 min on the ex vivo corneas for sampling metabolites on the surface over 9 days after burn with different concentratins of H2O2.
Abscissa: time scale [hours/days after burn]; ordinate: concentrations of glucose and lactate [mmol/l]. Each symbol stands for means +/- STD (n=3); the vertical line at 48 hours marks the exposure of the ex vivo corneas to H2O2.

In the supernatant rinsing medium of the same experiments with H_2O_2 burnt corneas the mean values of glucose and lactate levels did not change that much, and the standard deviations were rather high.

Dose response glucose/lactate metabolism in TCA treated corneas

Perfusion medium

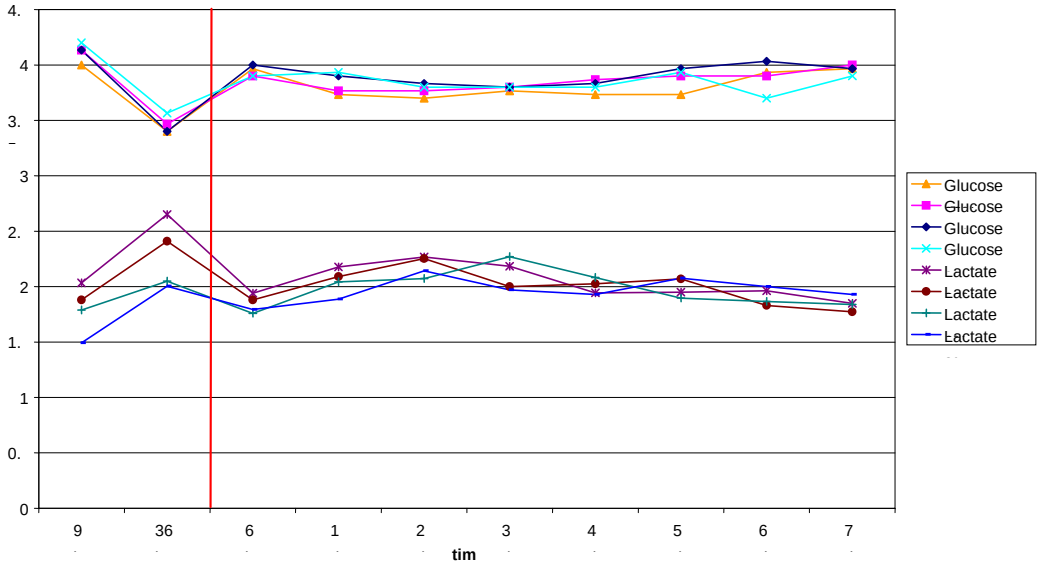

Fig. 38: Analyses of the perfusion media from ex vivo corneas over 9 days after burn with different concentrations of TCA.

Abscissa: time scale [hours/days after burn]; ordinate: concentrations of glucose and lactate [mmol/l]. Each symbol stands for means +/- STD (n=3); the vertical line at 48 hours marks the exposure of the ex vivo corneas to TCA.

After exposure of the ex vivo corneas to TCA, in the perfusion medium the glucose levels remained constantly on levels of about 4.0 mmol for the whole time of the experiment lasting 9 days. No differences were found, when various concentrations of TCA were applied. After the exposure, also the lactate levels did not decrease from 2.0 mmol. Thus, the glucose and lactate levels were consistent with a normal glucose turnover in the ex vivo corneas after irritation by TCA. Indeed, the biochemical results of TCA damage to the cornea resembled the diagrams of glucose/lactate metabolism of the untreated corneas.

Supernatant medium

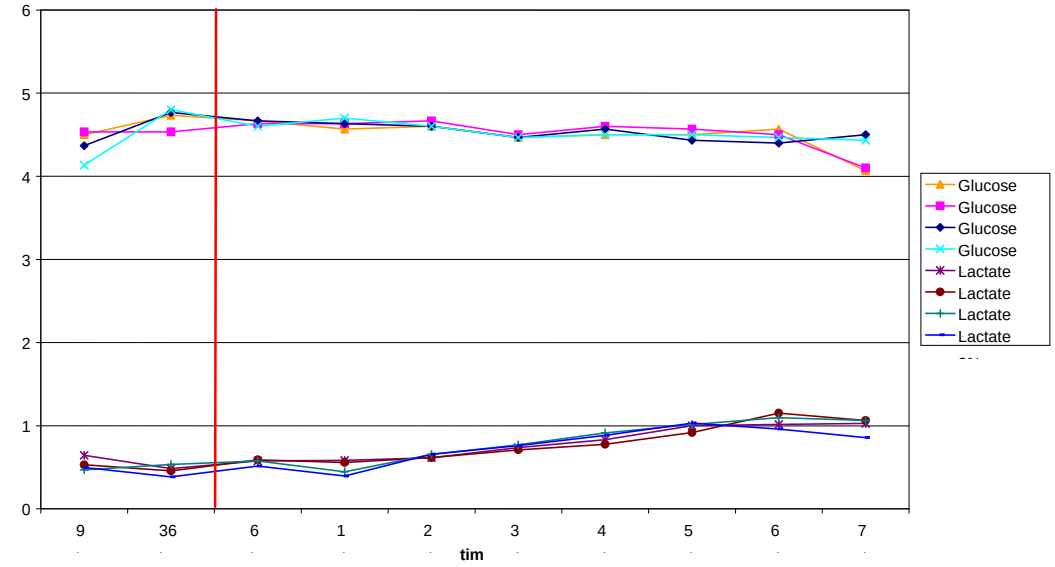

Fig. 39: Analyses of the supernatant medium rinsed for 15 min on the ex vivo corneas for sampling metabolites on the surface over 9 days after burn with different concentrations of TCA.
Abscissa: time scale [hours/days after burn]; ordinate: concentrations of glucose and lactate [mmol/l]. Each symbol stands for means +/- STD (n=3); the vertical line at 48 hours marks the exposure of the ex vivo corneas to TCA.

In the supernatant rinsing medium the glucose concentrations were constant, and the lactate increased only a little after burn with TCA just like in the untreated cornea as demonstrated in Fig. 23. There was no difference found in the various concentrations.

Dose response glucose/lactate metabolism in SLS treated corneas

Perfusion medium

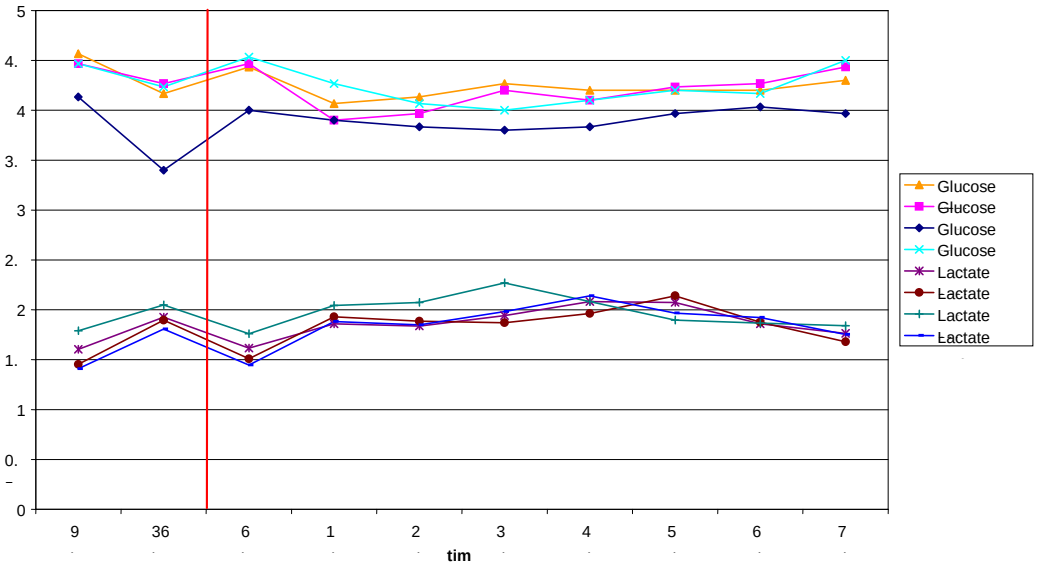

Fig. 40: Analyses of the perfusion media from ex vivo corneas over 9 daysafter burn with different concentrations with SLS.

Abscissa: time scale; the vertical line at 48 hours marks the exposure of the ex vivo corneas to SLS. Ordinate: concentrations of glucose and lactate in mmol. Each symbol stands for means +/- STD (n=3)

After exposure to SLS, the glucose and lactate levels in the perfusion medium were found to be very similar to the results obtained after exposure to TCA, i.e. they remained nearly constant with a little decrease in lactate production and glucose consumption several hours after burn. No dose dependence could be detected when observing the lactate production, an obvious lower level of the glucose concentration throughout the testings could be seen in the corneas treated with SLS 1,5%. The other concentrations of the chemical did not reveal a significant difference in the glucoe consumption.

Supernatant medium

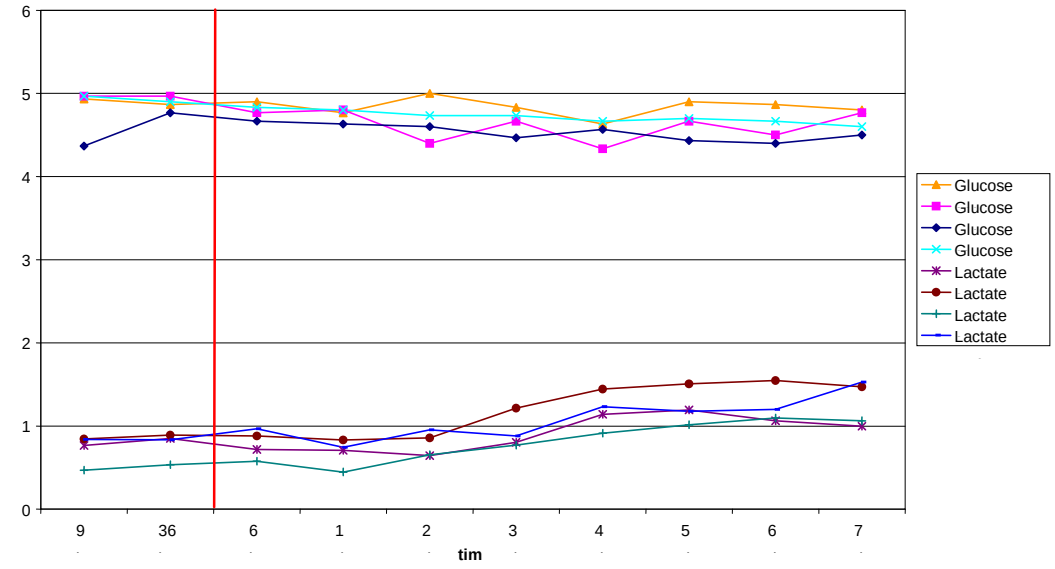

Fig. 41: Analyses of the supernatant medium rinsed for 15 min on the ex vivo corneas for sampling metabolites on the surface over 9 days after burn with different concentrations with SLS.
Abscissa: time scale [hours/days after burn]; ordinate: concentrations of glucose and lactate [mmol/l]. Each symbol stands for means +/- STD (n=3); the vertical line at 48 hours marks the exposure of the ex vivo corneas to SLS

An increase of the lactate production of the SLS burnt corneas could be seen from day 3 on after burn, but no clear dose response was to be detected, especially the higher concentrations did not lead to a higher break-in in the metabolic system.

Detection of cytokines and growthfactors in the perfusion medium

In the following diagrams the measured levels of several cytokines and growthfactors (IL-1α, IL-1β, IL-8, IL-10, MCP-1, TNFα, MIP-1β, FGF, VEGF) in untreated corneas and in those after burn with different chemicals (H_2O_2 3%, NaOH 2mol, Tomadol E14-5 10%) are reported. Each detected level represents the mean concentration in a pool of eight sampled perfusion media of the corneas.

Detection of IL1 alpha from pooled media samples

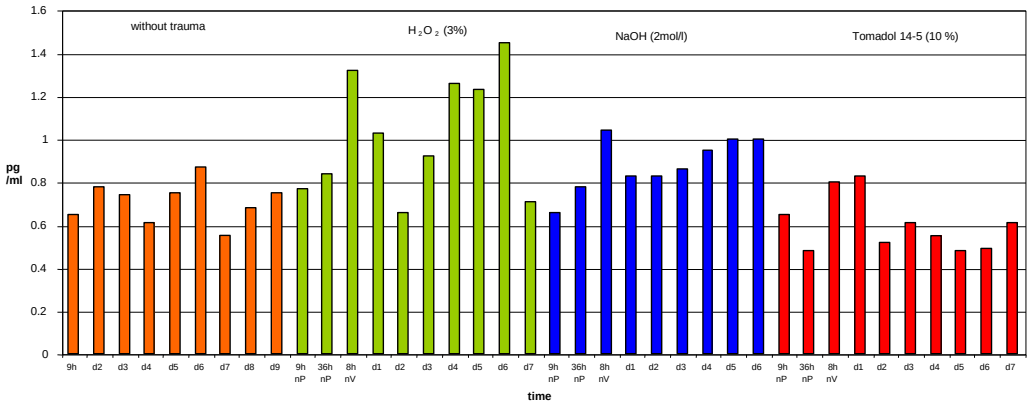

Fig. 42: Levels of IL-1-alpha after burn with different chemicals;
Abscissa: time scale for each of the exposure experiments; Ordinate: levels of Il-1-alpha in pg/ml of perfusion media.; nP = "after preparation"; nV = "after burn"

In the perfusion medium from ex vivo corneas without trauma, the IL-1-alpha appeared within the limits of reliable analyses after 2, 3, 5, 6, and 9 days. Compared to media from corneas without trauma, IL-1-alpha was increased by H_2O_2 exposure and after sodiumhydroxide. Lower levels resulted from Tomadol E14-5, hardly exceeding the lower limit of detection only at 8 hours and one day after exposure.

Detection of IL 1-beta from pooled media samples

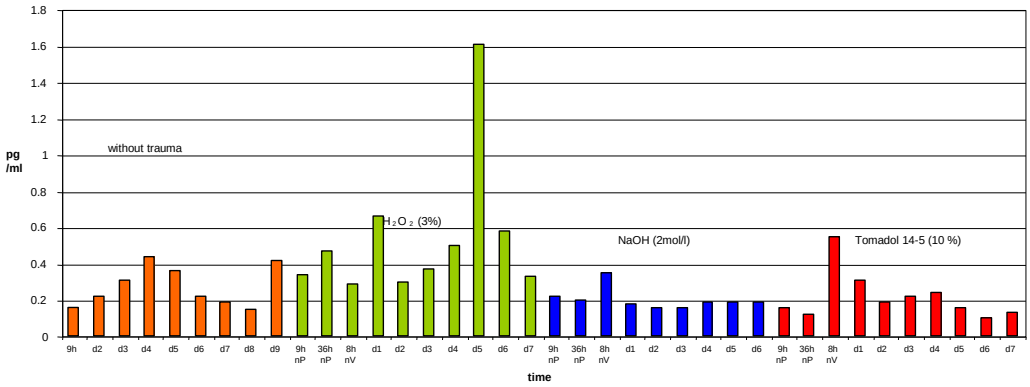

Fig. 43: Levels of IL-1-beta after burn with different chemicals;
Abscissa: time scale for each of the exposure experiments; Ordinate: levels of Il-1-beta in pg/ml of perfusion media.; nP = "after preparation"; nV = "after burn"

In the perfusion medium from ex vivo corneas without trauma, the IL-1-beta appeared within the limits of reliable analyses after 4 and 5 days. Compared to corneas without trauma, IL-1-beta is upregulated already 36 hours after preparation and on day 1, 3, 4 and 5 after H_2O_2 exposure with increasing levels. IL-1-beta exceeded the limit of detection only 8 hours after exposure to sodiumhydroxide and Tomadol E14-5.

Detection of IL-8 from pooled media samples

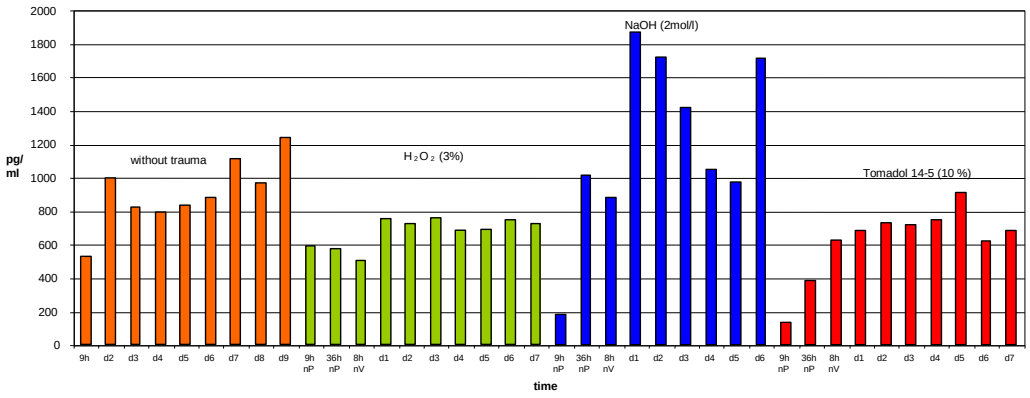

Fig. 44: Levels of IL-8 after burn with different chemicals;
Abscissa: time scale for each of the exposure experiments; nP = "after preparation"; Ordinate: levels of IL-8 in pg/ml of perfusion media.; nV = "after burn"

IL-8 appeared in all of the media samples clearly above the lower limit of detection. In the perfusion medium from ex vivo corneas without trauma, the levels of IL-8 were very prominent. Compared to corneas without trauma, IL-8 was rather low after exposure to H_2O_2. IL-8 is well represented already 36 hours after preparation and markedly elevated after exposure to 2n NaOH after 1, 2, and 3 days with decreasing levels and with a second maximum value on day 6. After exposure to tomadol E14-5, IL-8 exceeded generally in comparison to the initial values, but did not achieve the levels released from corneas without trauma.

Detection of MIP1-beta from pooled media samples

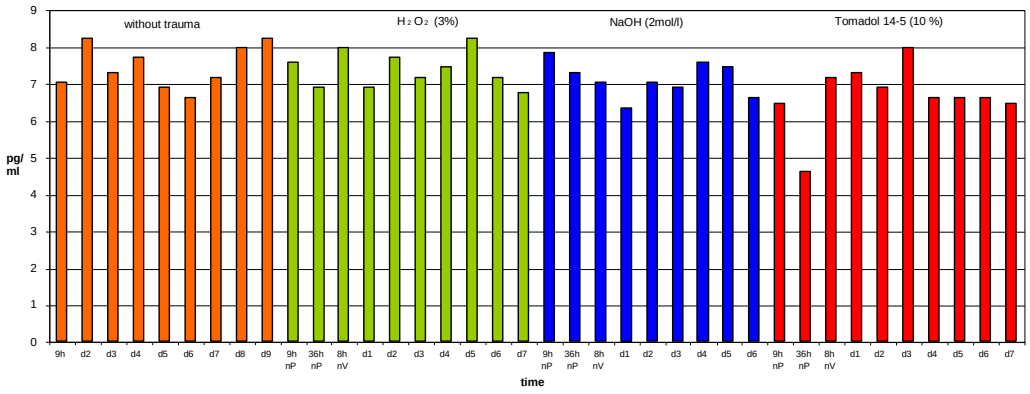

Fig. 45: Levels of MIP1-beta after burn with different chemicals;
Abscissa: time scale for each of the exposure experiments; Ordinate: levels MIP1-beta in pg/ml of perfusion media.; nP = "after preparation"; nV = "after burn"

MIP-1-beta appeared in all of the medium samples clearly above the lower limit of detection and in the same range of concentrations. Up to now, a different pattern of MIP-1-beta levels was not detectable.

Detection of basic FGF from pooled media samples

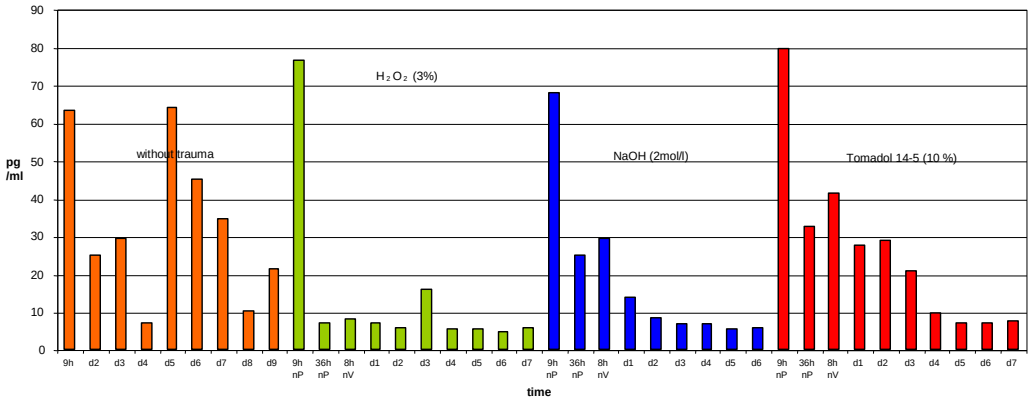

Fig. 46: Levels of FGF after burn with different chemicals;
Abscissa: time scale for each of the exposure experiments; nP = "after preparation"; Ordinate: levels of FGF in pg/ml of perfusion media.; nV = "after burn"

In all of the experiments presented in this diagram, FGF appeared in the perfusion medium with very high levels immediately after preparation, i.e. at 9 hours "nP". In the perfusion medium from ex vivo corneas without trauma, elevated levels of FGF were found at almost all times of the experiment. Compared to corneas without trauma, after exposure to H_2O_2, FGF remained rather low following the initial peak. After exposure to 2 n NaOH, the FGF level in the perfusion medium was increased only at 8 hours and then faded away. Before and after exposure to tomadol E14-5, following the initial peak value, FGF levels in the perfusion medium from the ex vivo corneas were elevated up to day 1, then gradually disappeared.

Detection of VEGF-165 from pooled media samples

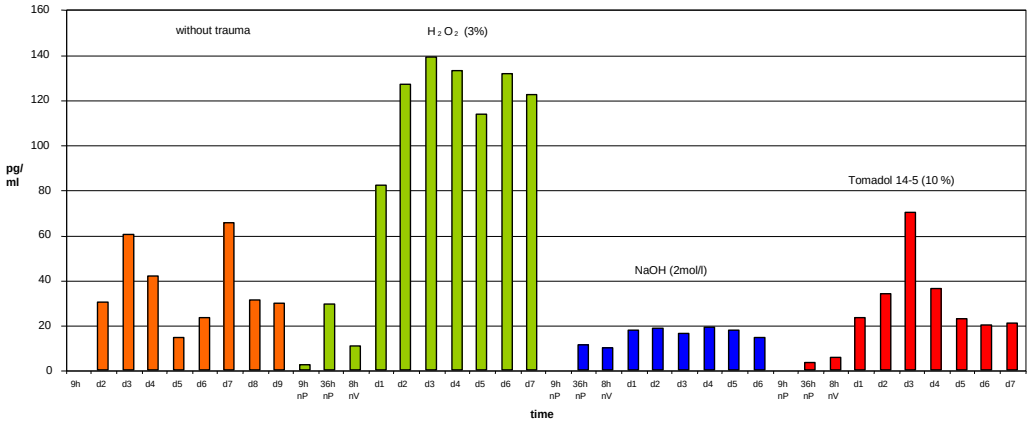

Fig. 47: Levels of VEGF-165 after burn with different chemicals;
Abscissa: time scale for each of the exposure experiments; Ordinate: levels of VEGF-165 in pg/ml of perfusion media.; nP = "after preparation"; nV = "after burn"

VEGF-165 appeared in all of the samples clearly above the lower limit of detection. In the perfusion medium from ex vivo corneas without trauma, the levels of VEGF-165 varied between about 1.2pg up to about 6.5pg. VEGF-165 levels were markedly elevated after exposure to H_2O_2, up to more than twice the levels of the ex vivo cornea without trauma and over the whole time period of the experiment. In contrast to its response to H_2O_2, VEGF-165 was rather low before and after exposure to 2 n NaOH. After exposure to tomadol E14-5, VEGF-165 exceeded generally initial values, but not the levels of corneas without trauma.

Dose response experiments with analyses of cytokines/growth factors in the perfusion medium

The analyses of these mediators measured in the perfusion medium of the artificial anterior chamber (AACM) were performed with the Luminex assay in the laboratory of the Institute for Diabetological Research at Düsseldorf, as described previously. In the following presentations, all data measured with the system were referred and normalized to the individual pre-incubation value of each of the ex vivo corneas. By this procedure, the absolute differences between the starting points of individual corneas were eliminated. The controls, untargeted corneas, if available, were processed mathematically the same way. In the dose response files of each mediator, the analytical data were computed with reference to the mean concentrations taken from all preincubated 187 corneas being analyzed 36 hours of pre-incubation before any exposure. This calculation did not eliminate the absolute starting point dependencies of individual corneas, since these values were saved in the data set of pre-incubated ex vivo corneas. Table 6 gives the mean values and standard deviations of all data of cytokine levels collected from the individual perfusion medium in the artificial anterior chamber (AACM) after 36 hours of pre-incubation before exposures to chemical injuries or abrasion of the epithelium. These values were taken for reference to the changes in cytokine levels during the following experiments.

Tab. 6: mean values and standard deviations of cytokine levels in the perfusion medium of 187 ex vivo corneas

	FGF	IL-8	IL-1 beta	IL-1 alpha	VEGF	MIP 1beta
Mean	4.511	273.268	0.244	0.653	43.243	3.566
Standard -deviation	±9.120	±178.1087	±0.223	±0.555	±60.860	1.720

The following diagrams, representing the results of the dose response experiments, show the levels of the different mediators in relation to the initial values after preincubation for 36 hours, i.e immediately before exposure, (in %) in a logarithmic scale. Every substance was tested on 3 corneas; the measeurements were done in the perfusion medium as well as in the supernatant medium.

Dose response of Fibroblastic Growth Factor (FGF) after NaOH burn

Perfusion medium

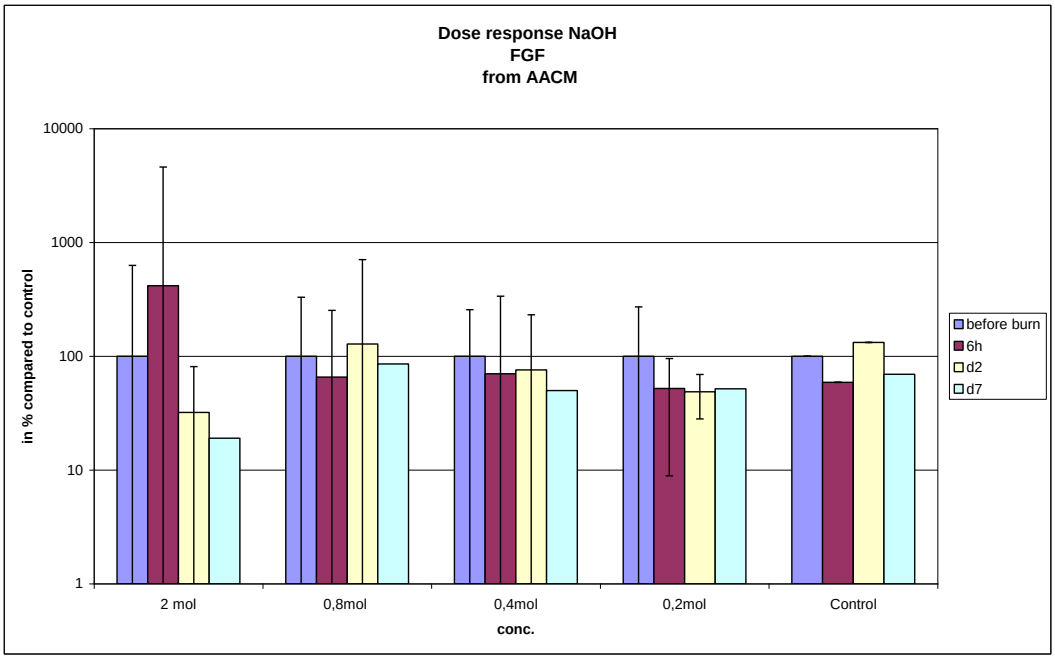

Fig. 48: Fibroblastic Growth Factor (FGF) levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of NaOH
Abscissa: various concentrations and time points; ordinate: procentual amount of FGF

When the ex vivo corneas were burnt with 0.2 mol, 0.4 mol and 0.8 mol NaOH, FGF concentrations in the perfusion medium did not differ from the initial values before exposure. But on exposure to 2 mol NaOH, FGF levels were increased by 500% six hours after the burn. However, standard deviations were excessively high.

Supernatant medium

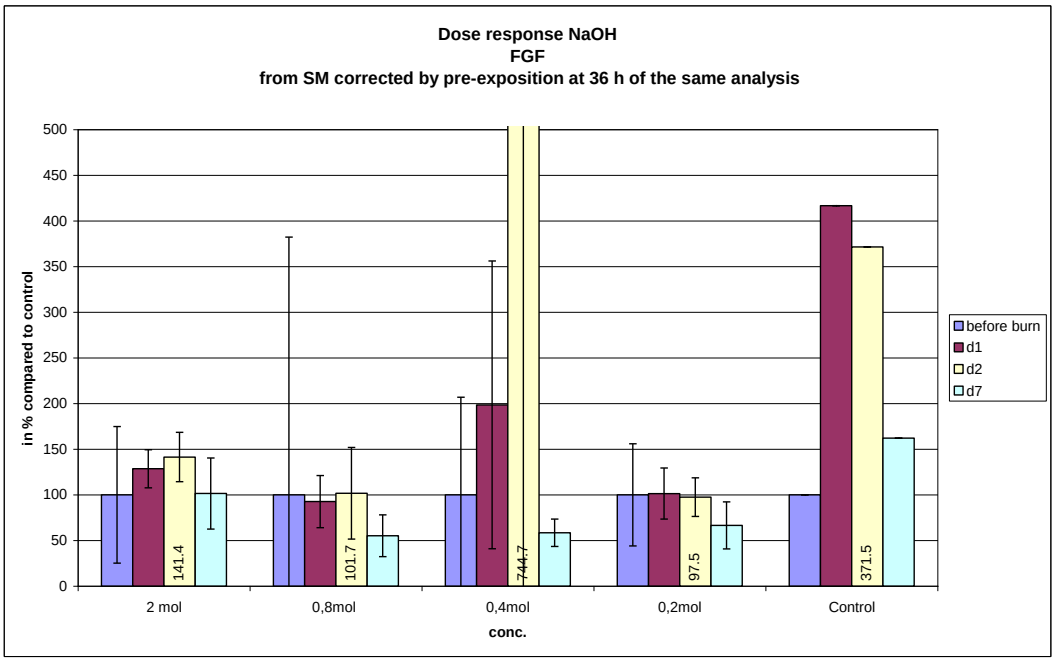

Fig. 49: Fibroblastic Growth Factor (FGF) levels in the supernatant rinsing medium (SM) of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of FGF

The results of this experiment show some excessive high FGF values on day 1 and 2 in the untouched control and two days after exposure to 0,4 mol NaOH. Apart from that no bigger alterations could be detected.

Dose response of Fibroblastic Growth Factor (FGF) after H2O2 burn

Perfusion medium

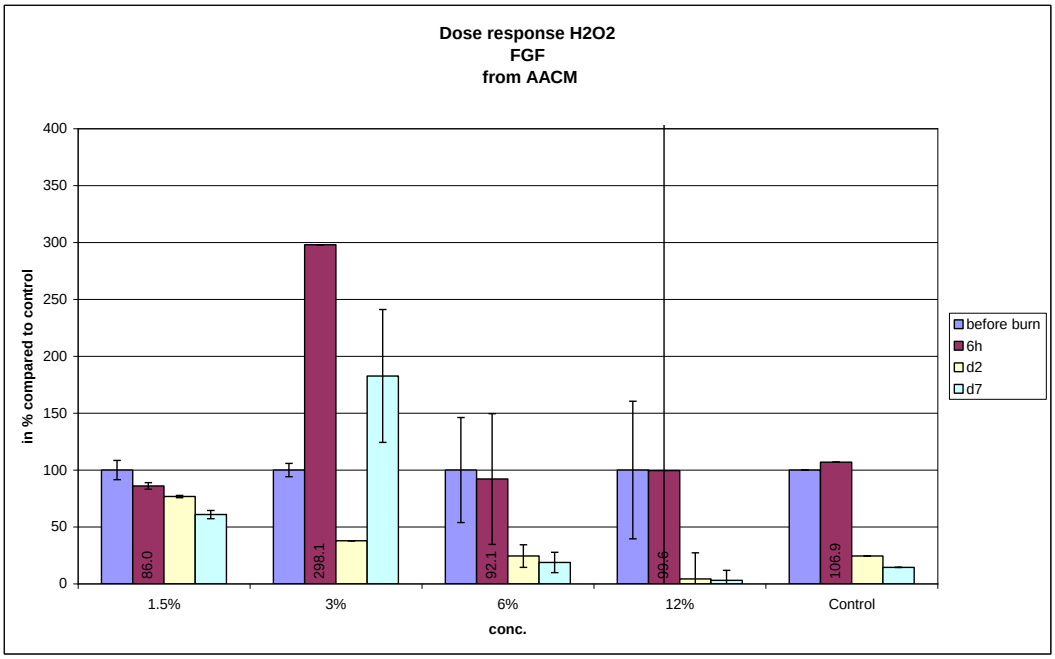

Fig. 50: Fibroblastic Growth Factor (FGF) levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of FGF

After application of H_2O_2 to the surface of the ex vivo corneas FGF release into the perfusion medium was different: With 1.5% H_2O_2 FGF decreased stepwise from 6 hours to two and further to seven days. On application of 3% H_2O_2, FGF was increased by 200% 6 hours later, and after 7 days still elevated by 80% of the initial values. After 6% H_2O_2, FGF was decreased to 25% and 20% of the initial values after 2 and 7 days, respectively. Two and seven days after exposure to 12% H_2O_2, FGF was almost disappeared from the perfusion medium. The same happened to untouched control corneas.

Supernatant medium

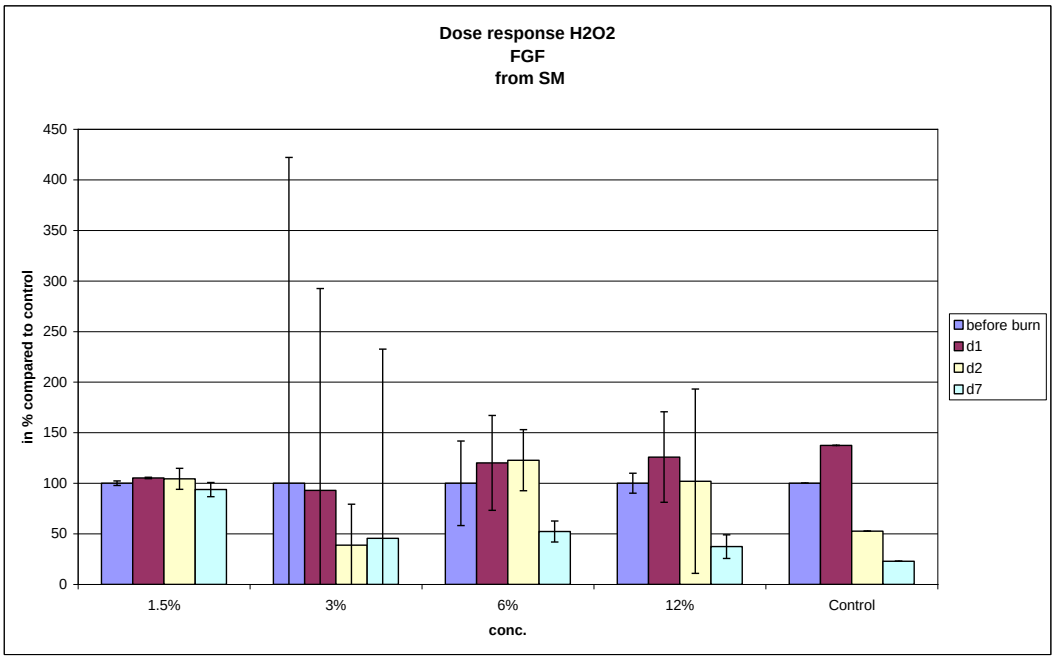

Fig. 51: Fibroblastic Growth Factor (FGF) levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of FGF

Like in the perfusion medium (AACM), in the supernatant rinsing medium (SM) FGF levels tended to be decreased after exposure to higher concentrations of H_2O_2. The very high standard deviations in the SM after exposure to 3% H_2O_2 resembled the prominent response in the perfusion medium.

Dose response of Fibroblastic Growth Factor after TCA burn

Perfusion medium

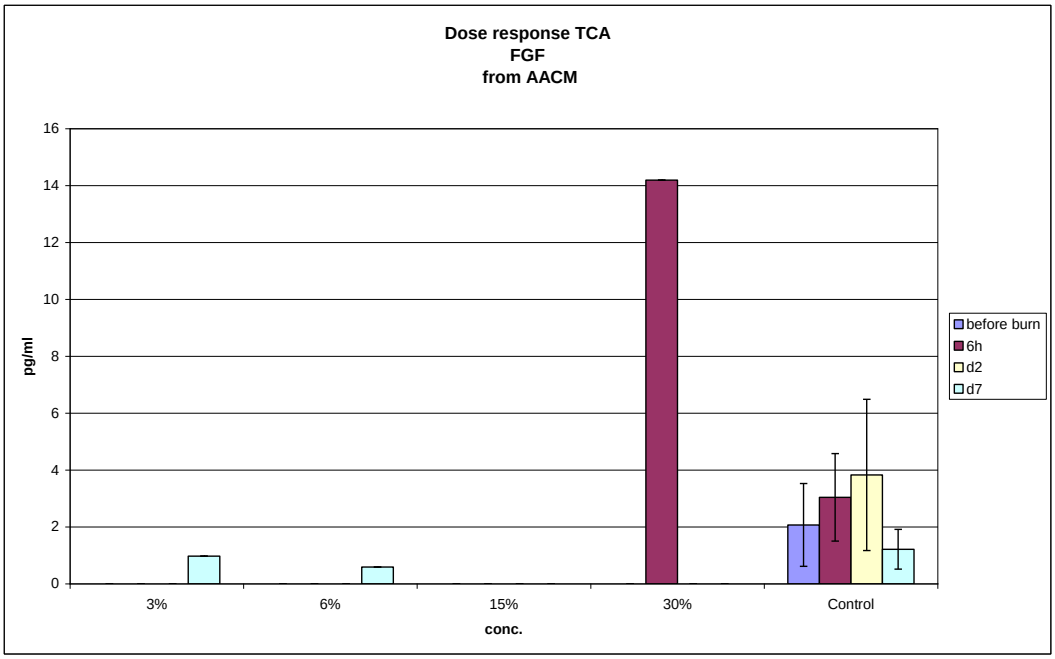

Fig. 52: Fibroblastic Growth Factor (FGF) levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of TCA
Abscissa: various concentrations and time points; ordinate: amount of FGF in pg/ml

In this and the follwing diagram for once the amount of FGF is noticed in absolute values in pg/ml, due to many measurement results out of range <0, below the lower detection limit. After burn with TCA no FGF could be detected in neither in the perfusion nor the supernatant medium, except one very high peak in the perfusion medium six hours after burn with 30% TCA.

Supernatant medium

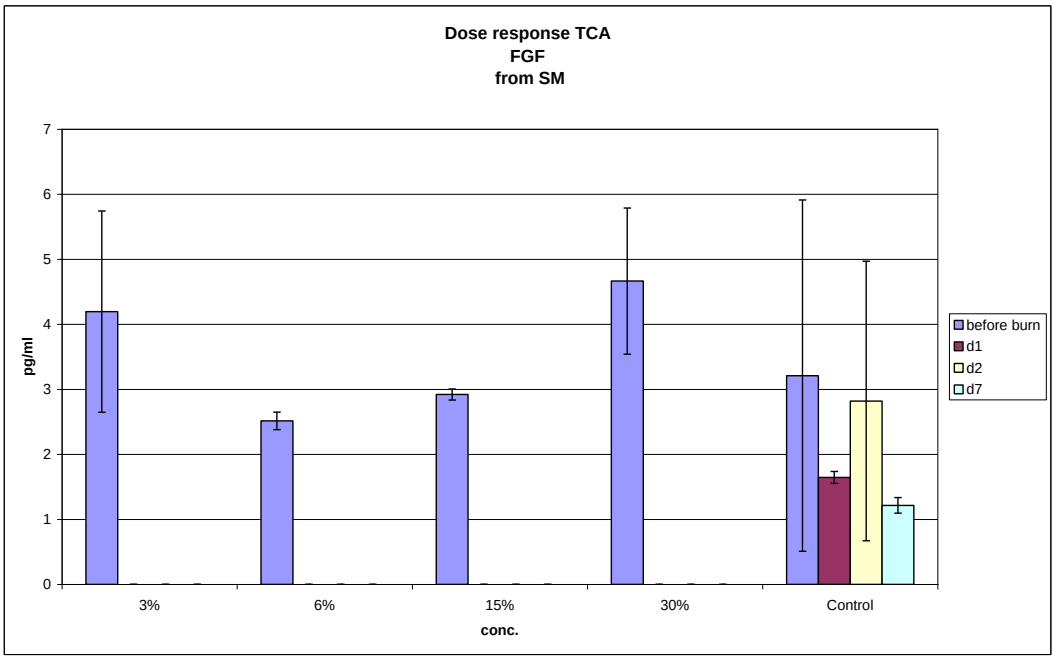

Fig. 53: Fibroblastic Growth Factor (FGF) levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: amount of FGF in pg/ml

Dose response of Fibroblastic Growth Factor (FGF) after SLS burn

Perfusion medium

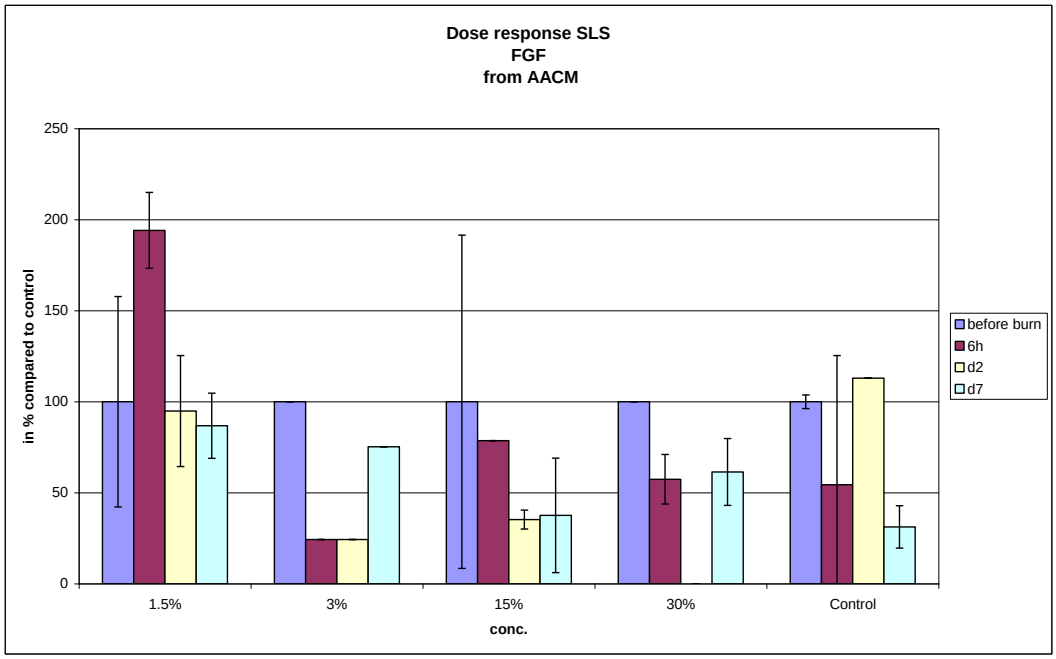

Fig. 54: Fibroblastic Growth Factor (FGF) levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of SLS
Abscissa: various concentrations and time points; ordinate: procentual amount of FGF

The experiments with 1.5% sodium lauryl sulfate (SLS) showed 6 hours after exposure a significant increase of FGF in the perfusion medium to almost double of the initial values. With application of higher concentrations of SLS to the surface of the ex vivo corneas, FGF release to the perfusion media was decreased.

Supernatant medium

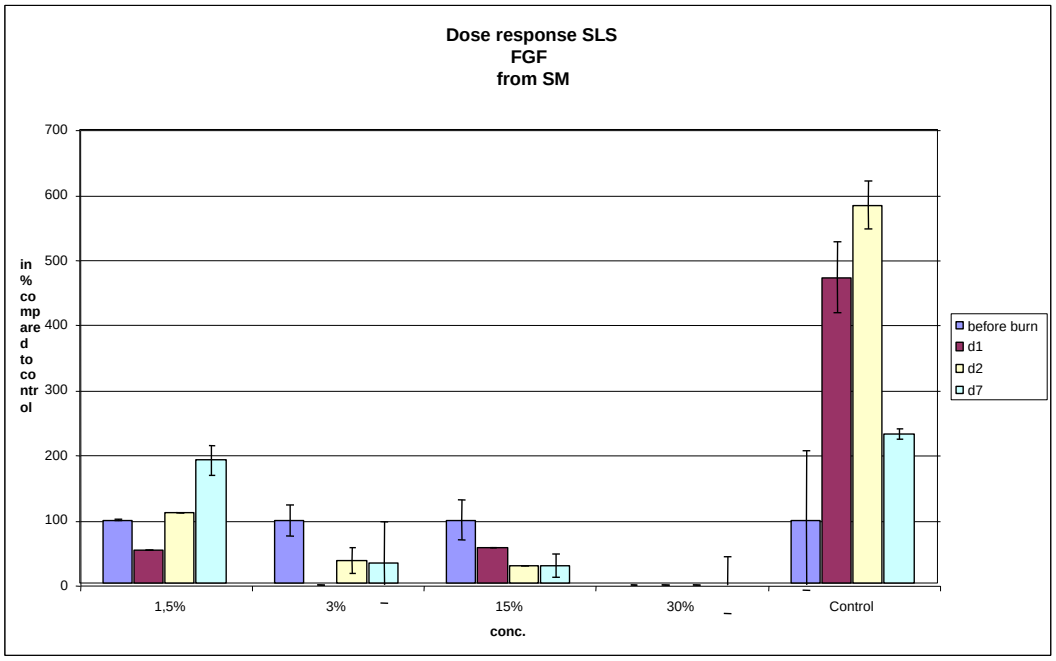

Fig. 55: Fibroblastic Growth Factor (FGF) levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of FGF

In the supernatant rinsing medium (SM), after SLS exposure FGF levels were rather low. Only with 1.5% SLS exposure FGF was significantly elevated after seven days. But control values were excessively high like in the supernatants after exposure to NaOH.

Dose response of IL-1α after NaOH burn

Perfusion medium

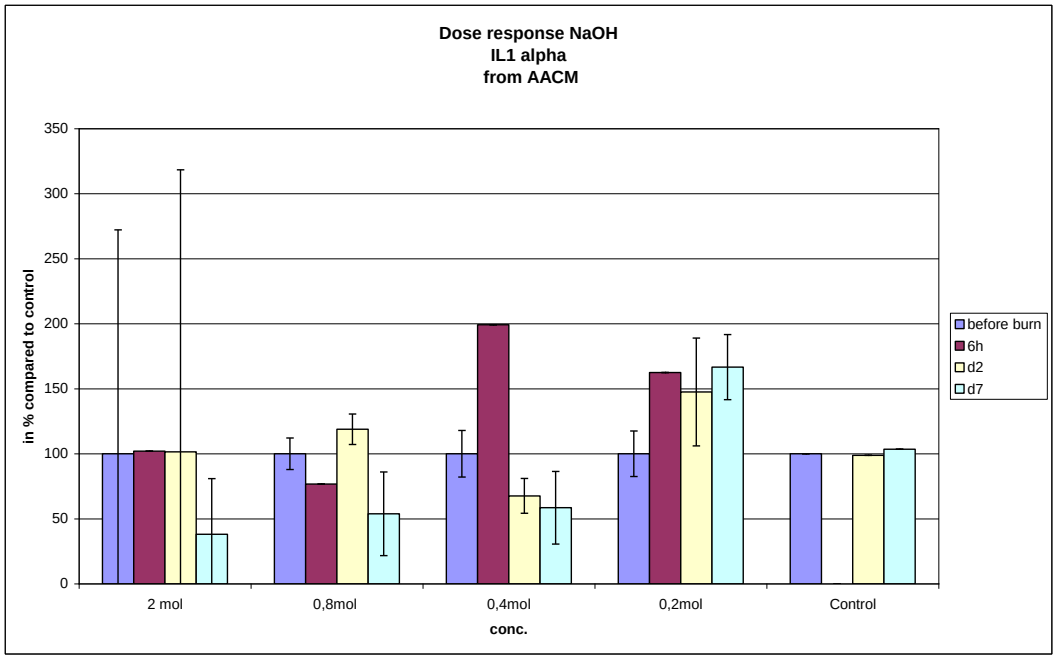

Fig. 56: IL-1α levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1α

In these dose response experiments on the time point of 6 hours after exposure IL-1-alpha was increased in the perfusion medium with exposure to 0.2 mol NaOH by 60% of the values before, and with 0.4 mol NaOH by 100%. Two and seven days after exposure to 0.2 mol NaOH, IL-1-alpha was still increased by 50% and 70%, respectively, while on the same time, after 0.4% NaOH, IL-1-alpha was significantly decreased. After exposure to 0.8 and 2.0 mol NaOH, the ex vivo corneas did not produce higher levels of IL-1-alpha in the perfusion medium than normally found in unexposed ones 6 hours and 2 days later. However, seven days after exposure to 0.8 and 2.0 mol NaOH IL-1-alpha levels were significantly decreased.

Supernatant medium

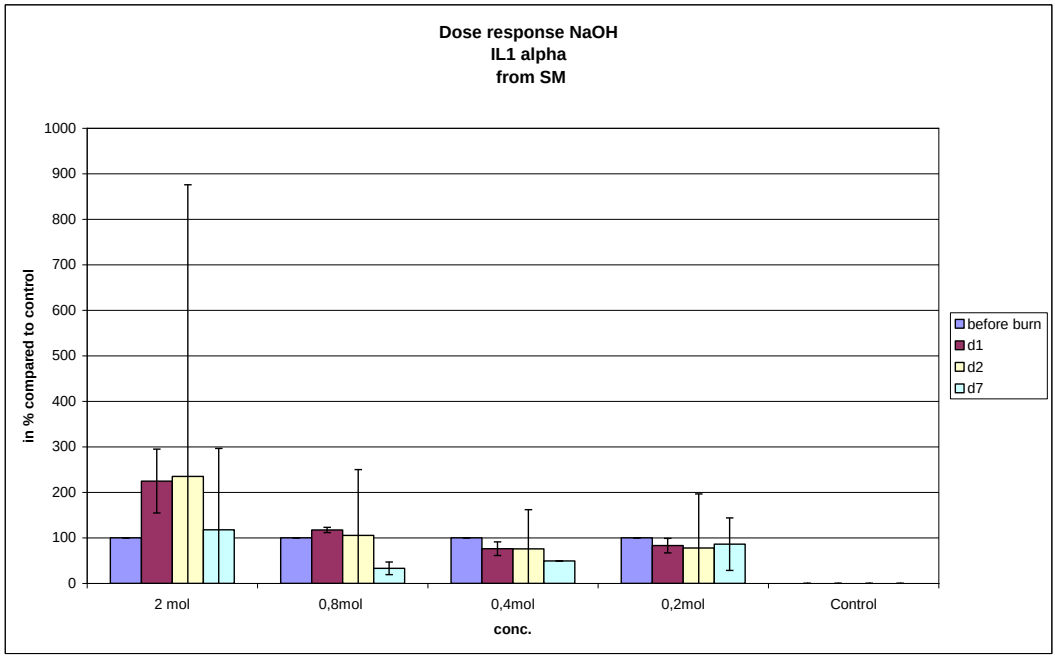

Fig. 57: IL-1α levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1α

In the supernatant rinsing medium after exposure to NaOH IL-1-alpha increased only, when 2 mol NaOH was applied. Then, one day afterwards, IL-1-alpha was elevated by 100% of the initial values. After exposure to 0.8 and 0.4 mol NaOH, IL-1-alpha was decreased, and after application of 0.2 mol NaOH IL-1-alpha remained unchanged.

Dose response of IL-1α after H_2O_2 burn

Perfusion medium

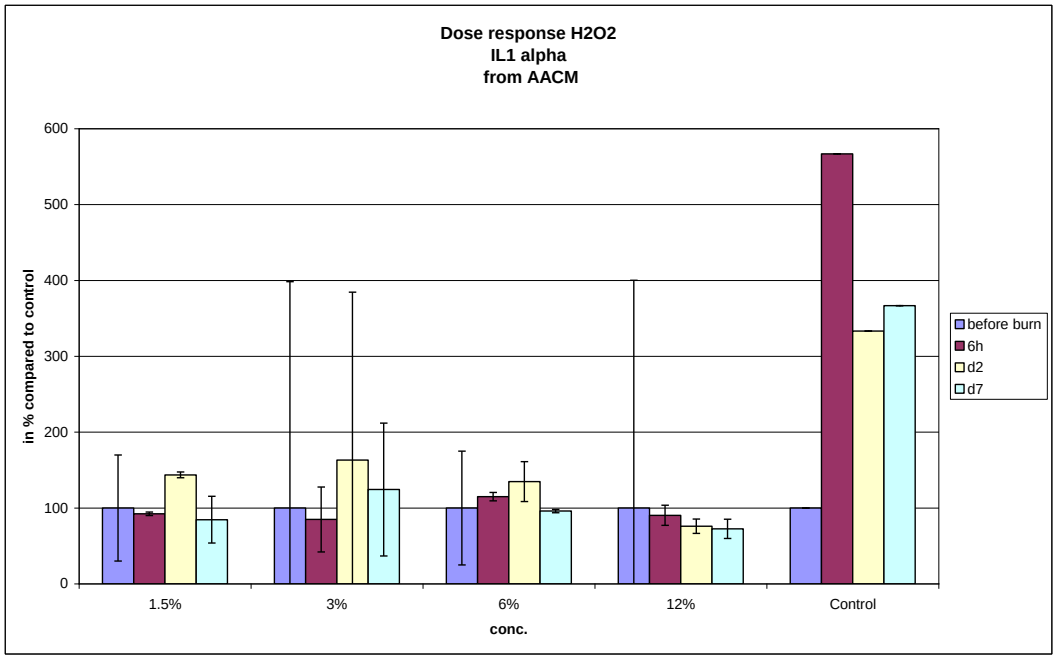

Fig. 58: IL-1α levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1α

After exposure to H_2O_2, the perfusion medium did not show important changes of the IL-1-alpha levels. There were some very high standard deviations in the experiments with 3% and 12% H_2O_2, and in the control values excessive IL-1-alpha levels.

Supernatant medium

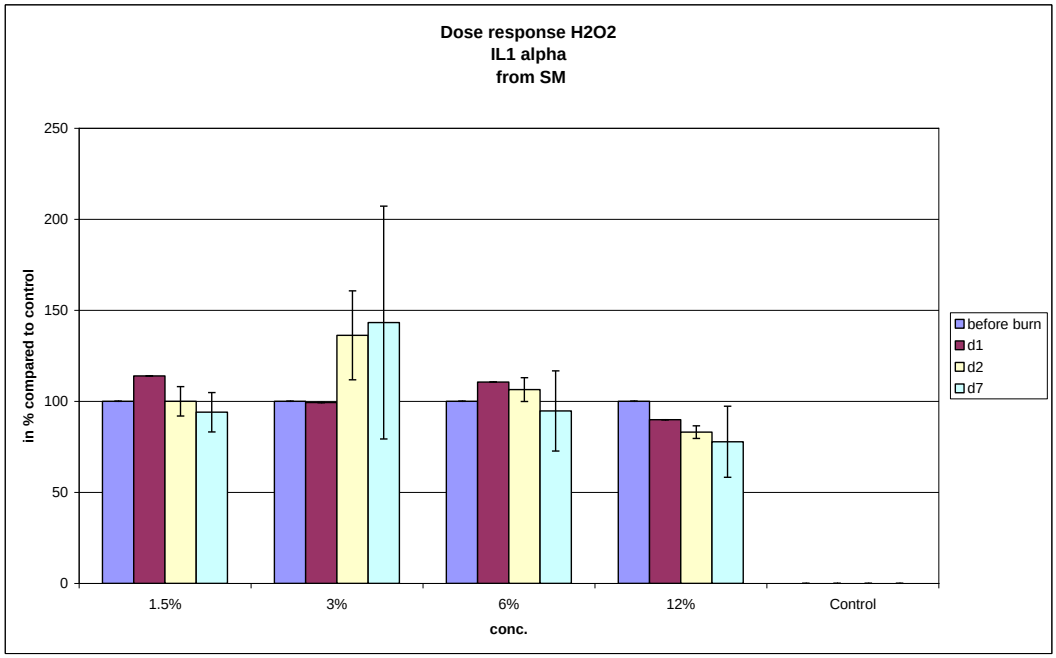

Fig. 59: IL-1α levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1α

In the supernatant rinsing medium after exposure to H_2O_2 no prominent deviations from normal values of il-1α were observed. However, two and seven days after 3% H_2O_2 exposure elevations of the IL-1-alpha levels were found by 70% and 80% from the initial values, which could be statistical significant on day 2, as estimated from standard deviations.

Dose response of IL- 1α after TCA burn

Perfusion medium

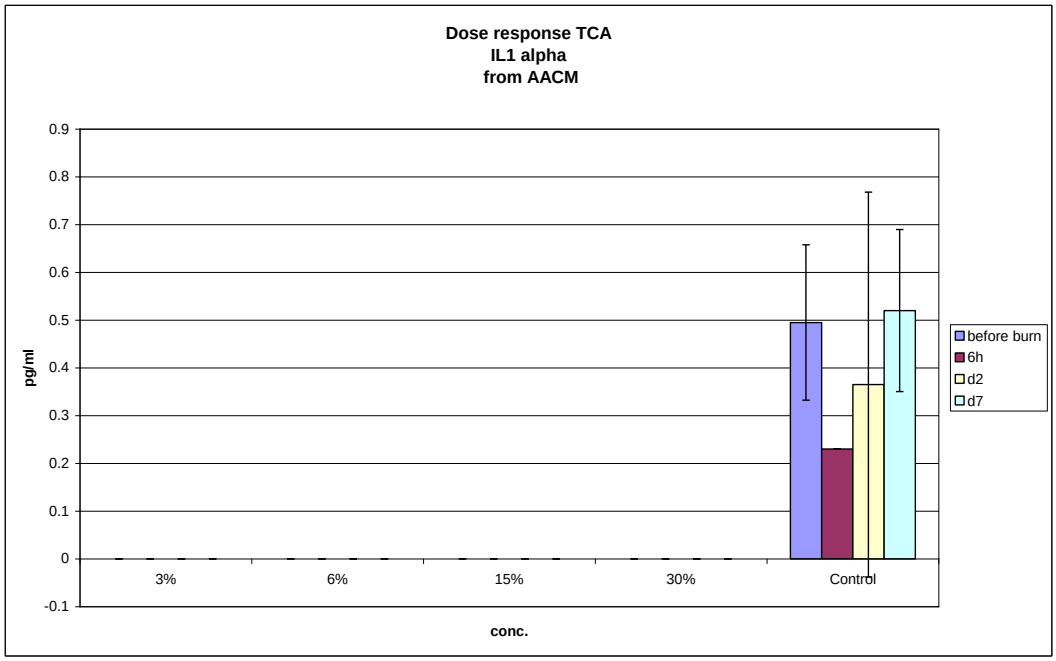

Fig. 60: IL-1α levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: amount of IL-1α in pg/ml

Supernatant medium

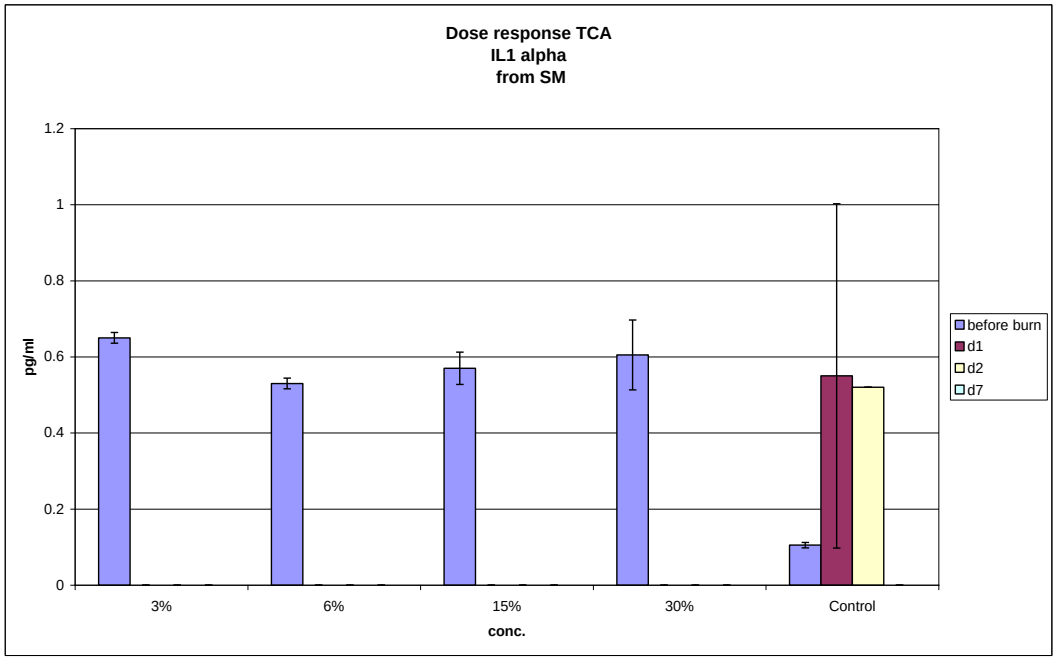

Fig. 61: IL-1α levels in the supernatant medium (AACM) of ex vivo corneas after exposure to various concentrations of TCA Abscissa: various concentrations and time points; ordinate: amount of IL-1α in pg/ml

After exposure to TCA no IL-1-alpha was detected neither in the perfusion nor the supernatant rinsing medium.

Dose response of IL-1α after SLS burn

As too many measurement results were out of range < 0, below the lower detection limit the follwing digrams do not use the logarithmic scale like in the previous diagrams but absolute values in pg/ml.

Perfusion medium

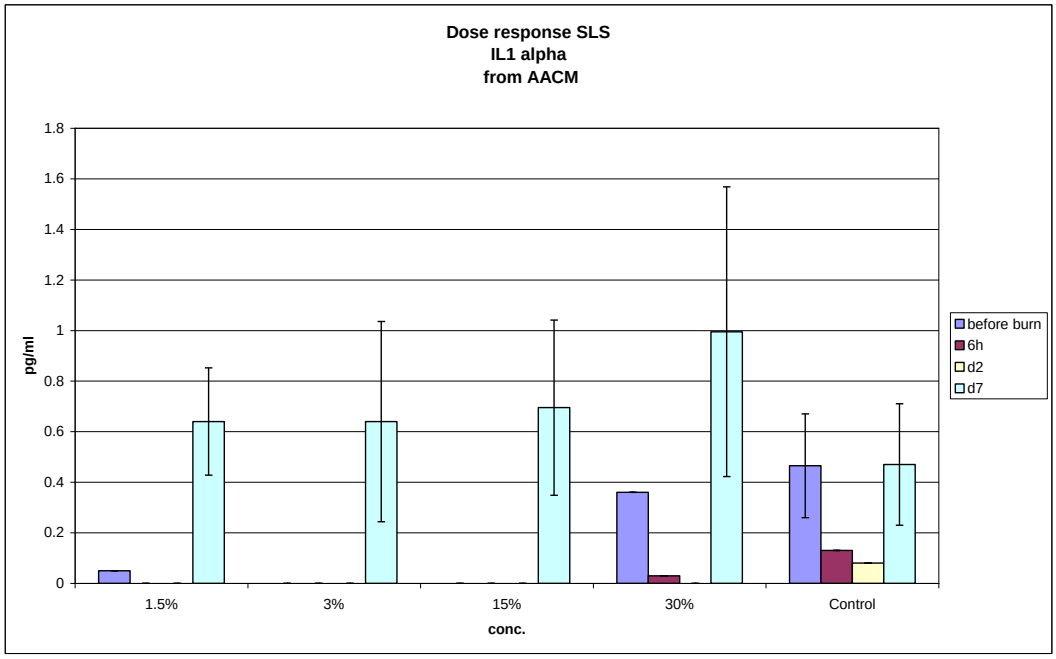

Fig. 62: IL-1α levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of SLS.

Abscissa: various concentrations and time points; ordinate: amount of IL-1α in pg/ml

Supernatant medium

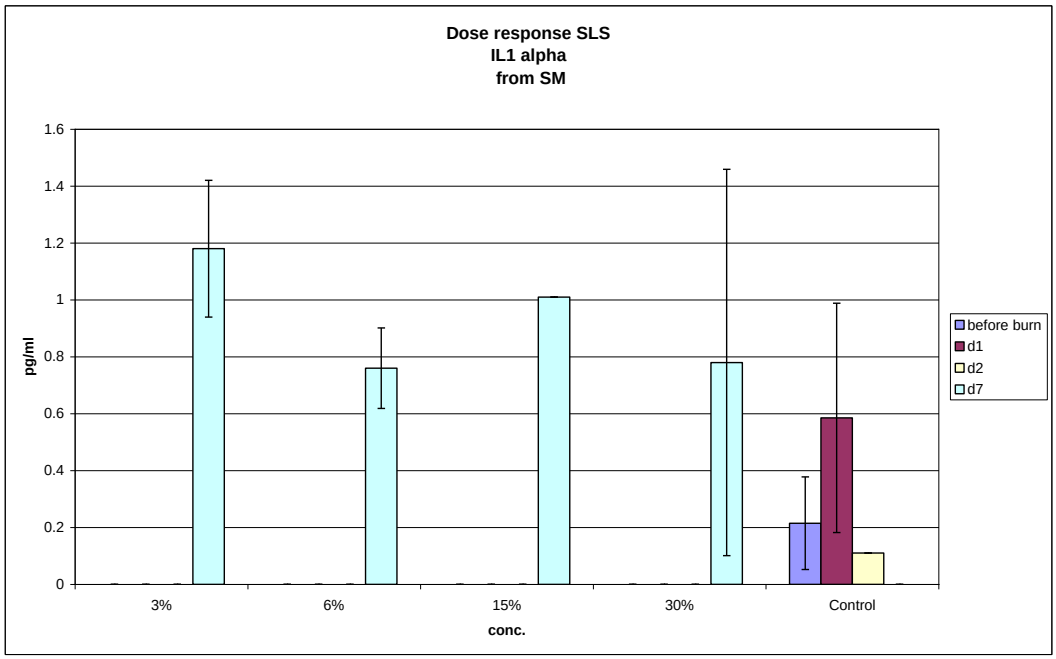

Fig. 63: IL-1α levels in the supernatant medium (AACM) of ex vivo corneas after exposure to various concentrations of SLS.

Abscissa: various concentrations and time points; ordinate: amount of IL-1α in pg/ml

In the perfusion medium as well as the supernatant medium in the first days after burn with SLS nearly no IL-1α could be detected, exept after burn witht SLS 30% in the perfusion medium after six hours. On day seven there were concentrations between 0.6 and 1.0 pg/ml in the perfusion medium respectively between 0.75 and 1.2 pg/ml measured. In the control group at the begin and after seven days values of 0.4pg/ml could be detected in the perfusion medium, after six hours and two days lower concentrations were found. The highest peak in the supernatant medium was measured after one day of about 0.6 pg/ml.

Dose response of IL-1β after NaOH burn

Perfusion medium

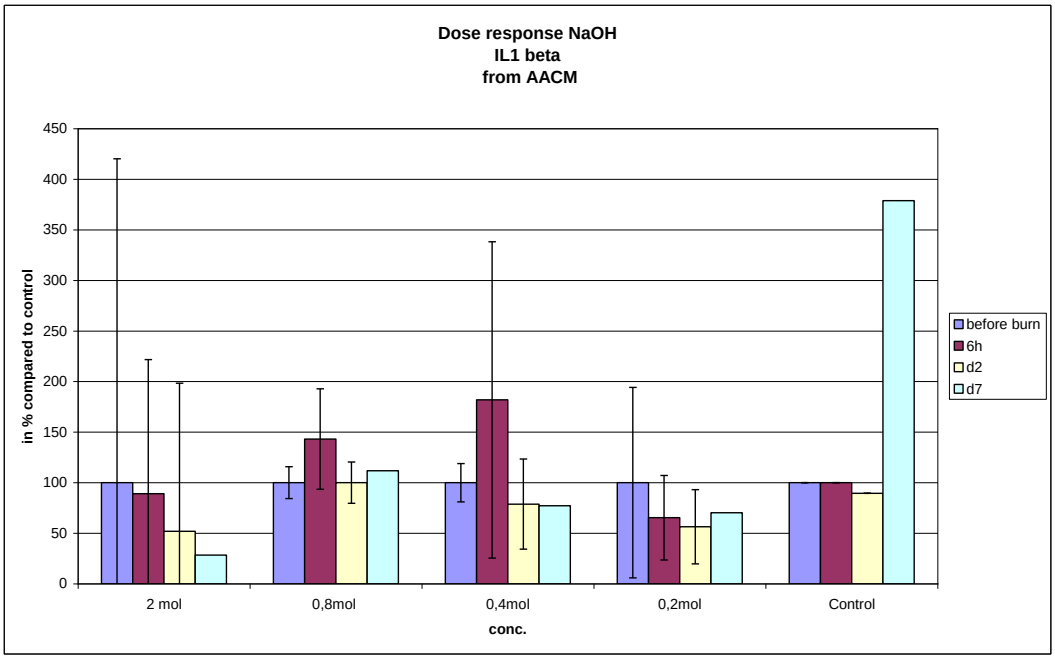

Fig. 64: IL-1β levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1β

The levels of IL-1-beta in the perfusion medium after exposure of the ex vivo cornea to NaOH resembled the results of IL-1-alpha analyses as presented in fig. 56. With IL-1-beta, the same increase was observed 6 hours after exposure to 0.4 mol NaOH. Contrary to IL-1-alpha, IL-1-beta did not increase in the perfusion medium after exposure to 0.2 mol NaOH. In general, after exposure to NaOH concentrations as applied in these experiments, the tendency of decreased release was more prominent with IL-1-beta than with IL-1-alpha.

Supernatant medium

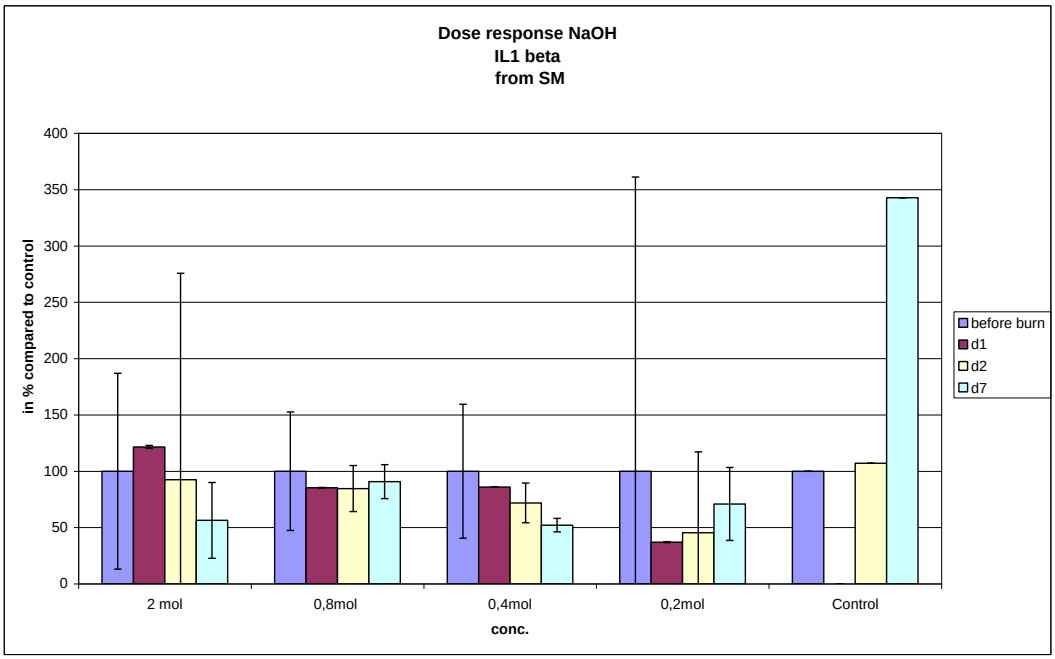

Fig. 65: IL-1β levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1β

Exept on day one after burn with NaOH 2mol there was a decrease in the IL-1β concentrations throughout the whole experiments in the supernatant medium after burn with the different concentrations of NaOH.

Dose response of IL-1β after H_2O_2 burn

Perfusion medium

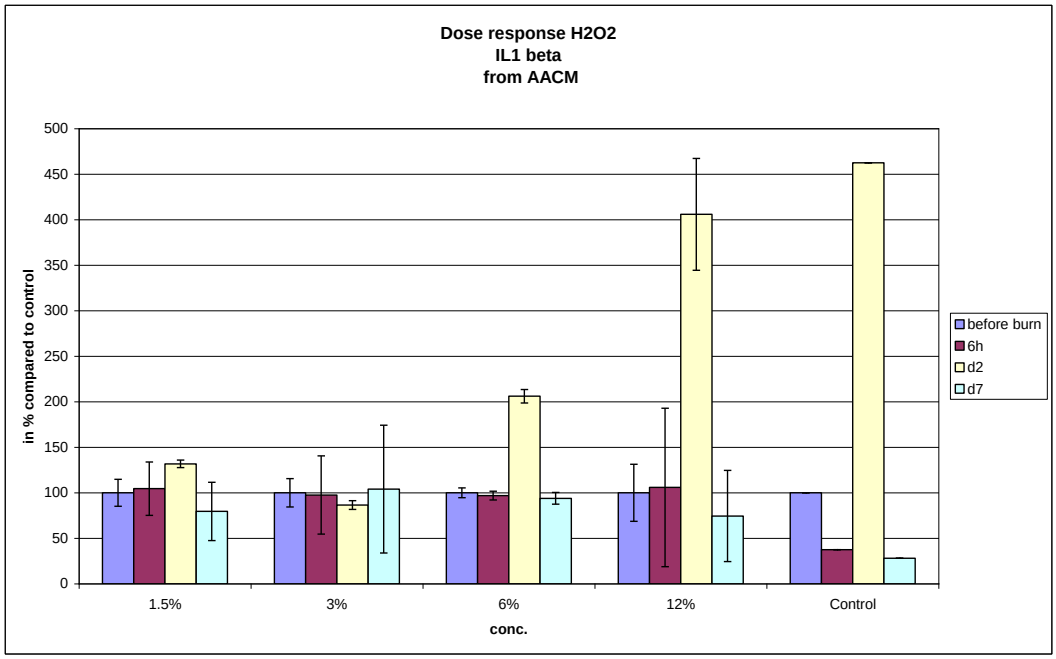

Fig. 66: IL-1β levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1β

After exposure to 6% and 12% H_2O_2 IL-1-beta was increased in the perfusion medium on day 2 by 100% and 300%, respectively, compared to the initial values before the experiment. However, the control series of ex vivo corneas, running in parallel, revealed a similar increase of IL-1-beta in the perfusion medium on the same day 2 of ex vivo cultivation. Apart from that nearly no variation in concentration of IL-1β was detected in the perfusion medium.

Supernatant medium

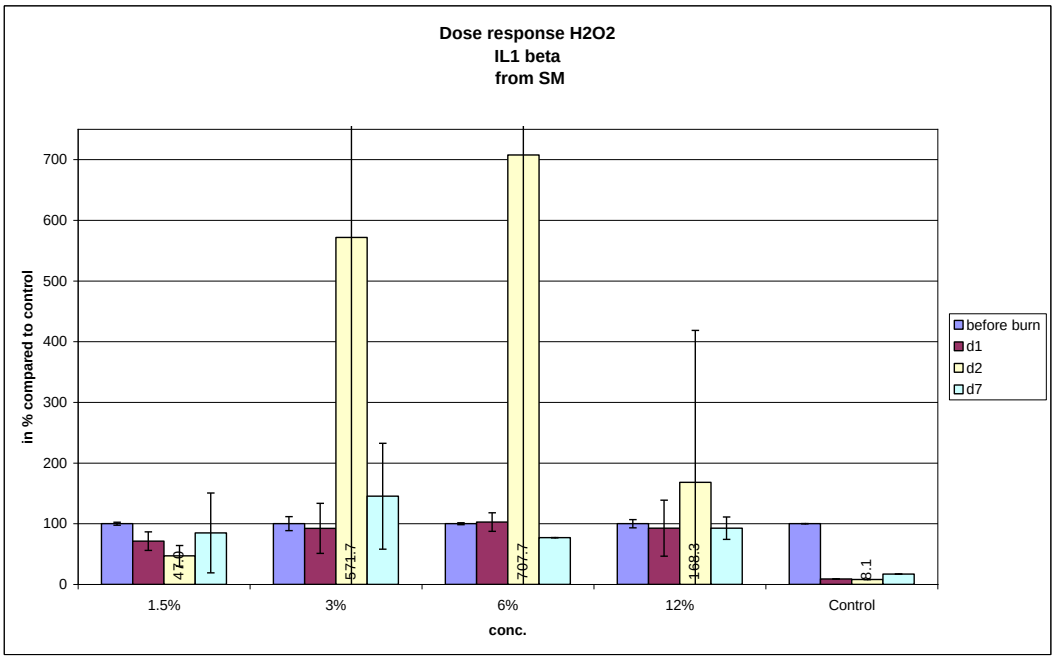

Fig. 67: IL-1β levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1β

On the second day after burn with H_2O_2 3% resp. 6% very high concentrations of IL-1-beta (570% resp. >700%) could be detected. The other measurements schowed rather normal values without any bigger in-/decreases.

Dose response of IL-1β after TCA burn

Perfusion medium

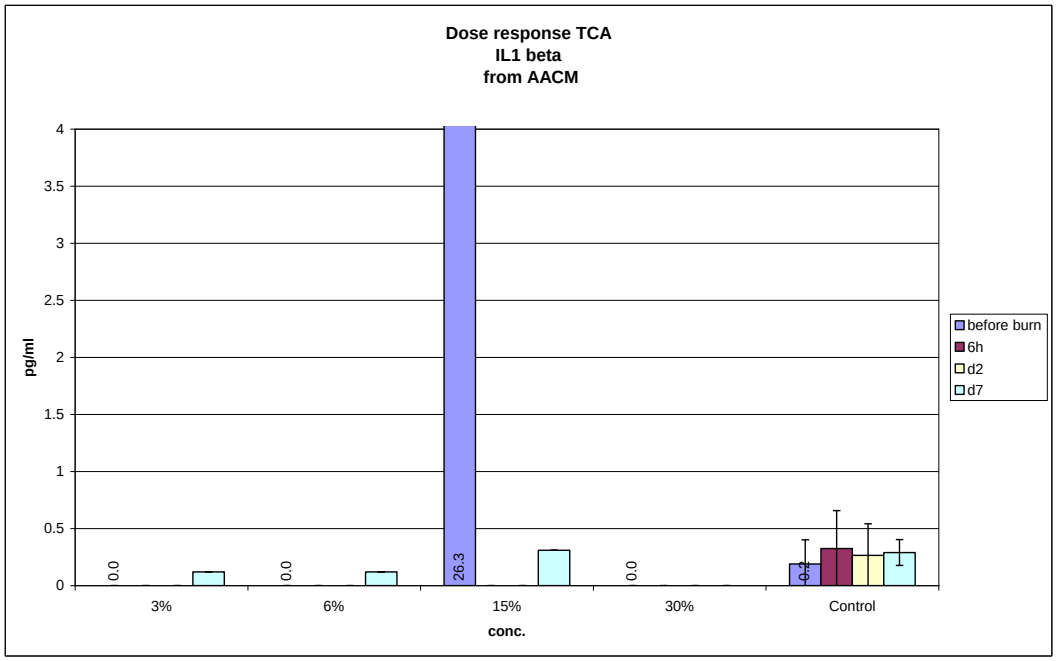

Fig. 68: IL-1β levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: amount of IL-1β in pg/ml

Supernatant medium

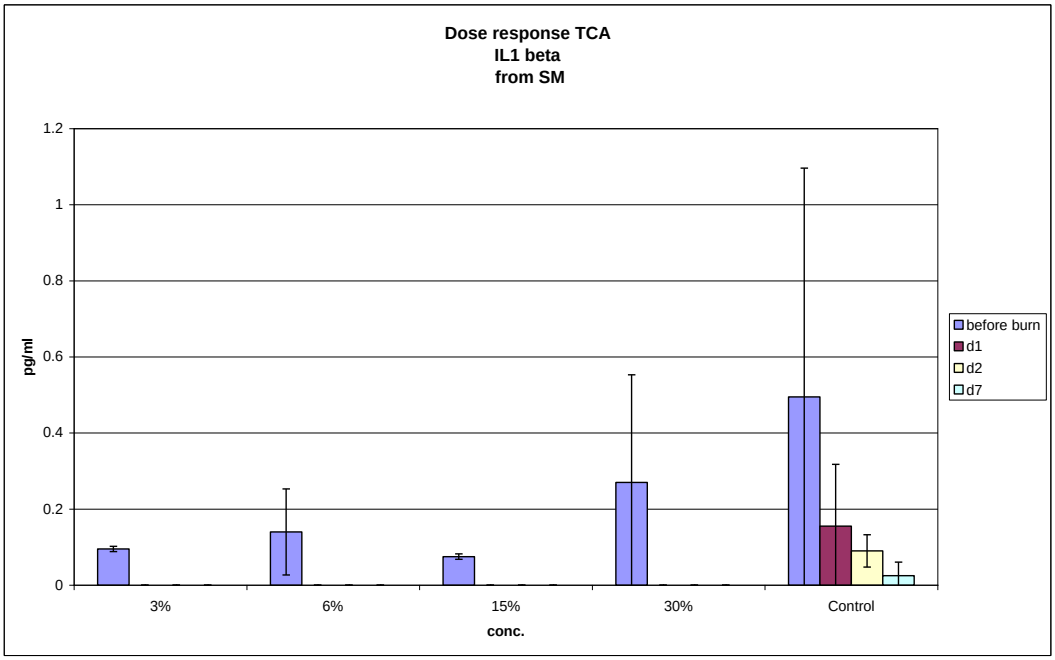

Fig. 69: IL-1β levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: amount of IL-1β in pg/ml

Like in the analyses of FGF and IL-1-alpha after the exposure to TCA no IL-1-abeta was detected in both media.

Dose response of IL-1β after SLS burn

Perfusion medium

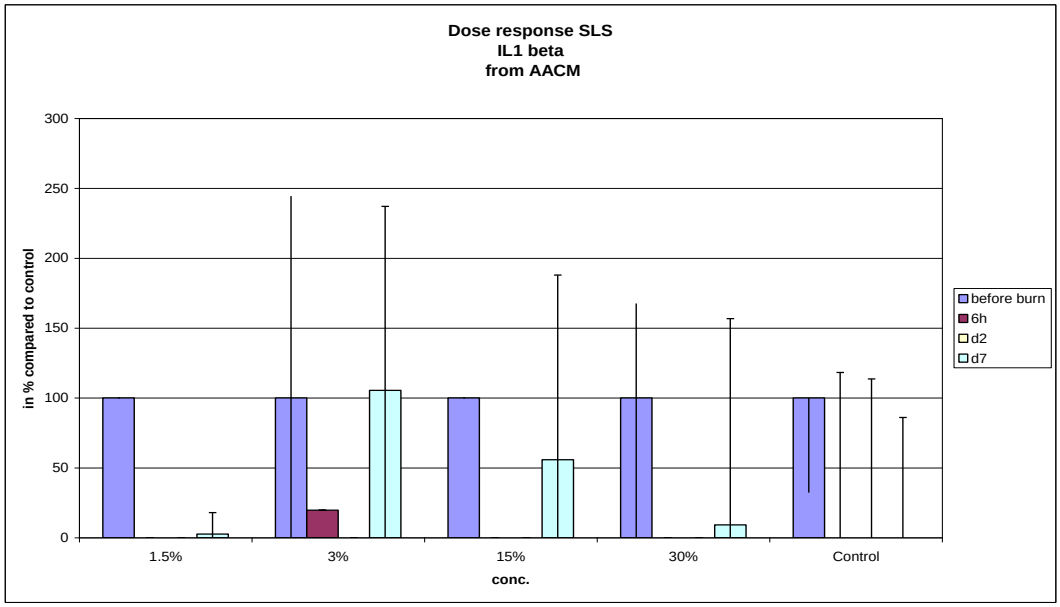

Fig. 70: IL-1β levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1β

Supernatant medium

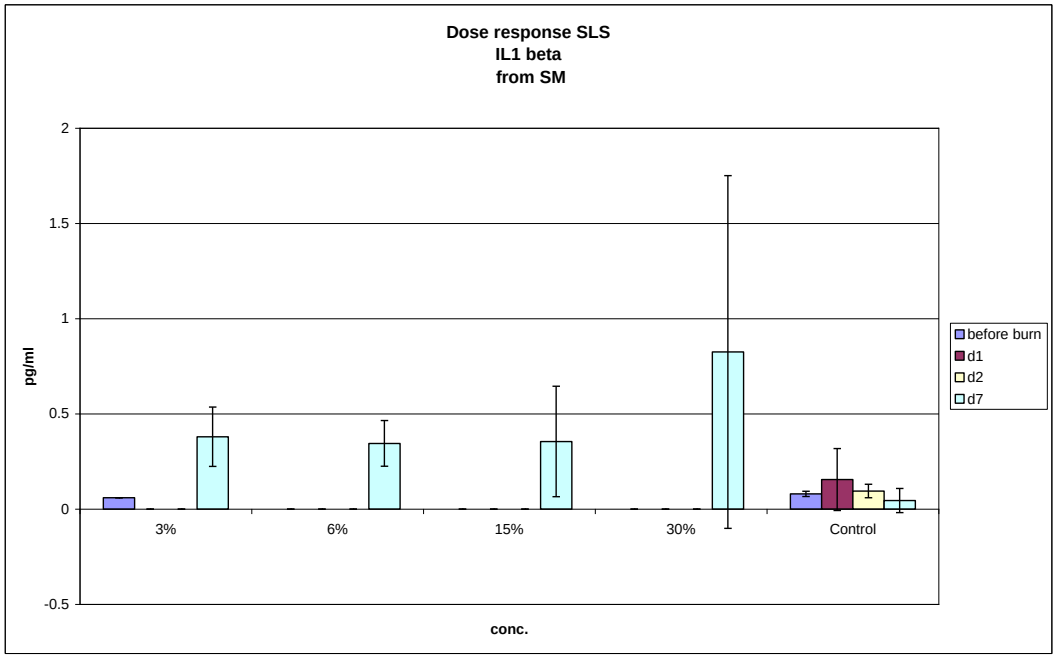

Fig. 71: IL-1β levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-1β

In both media (perfusion and supernatant) only on day seven after burn IL-1-beta could be detected in a significant way. Due to many measurement results out of range < 0, below the lower detection limit in the supernatant medium the levels in the digram are indicated in absolute values [pg/ml].

Dose response of IL-8 after NaOH burn

Perfusion medium

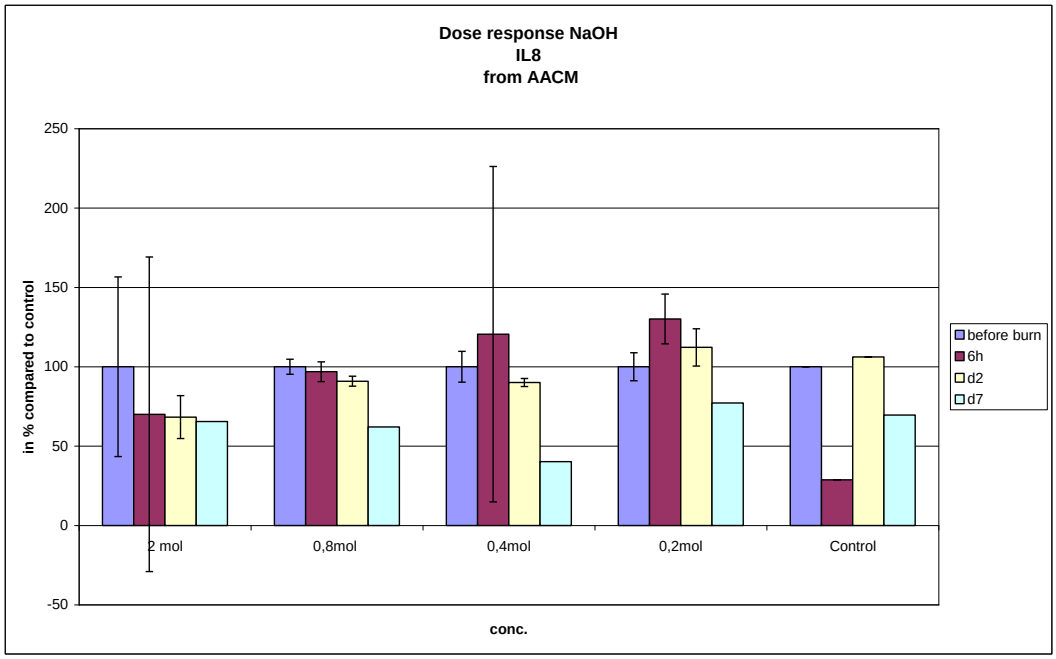

Fig. 72: IL-8 levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-8

After burn with relatively mild concentrations of NaOH (0.2 and 0.4 mol) there was an increase of the IL-8 concentration on the first days and afterwards a decrease. With higher concentrations no boost at all could be detected but a decrease in the IL-8 values from the beginning on.

Supernatant medium

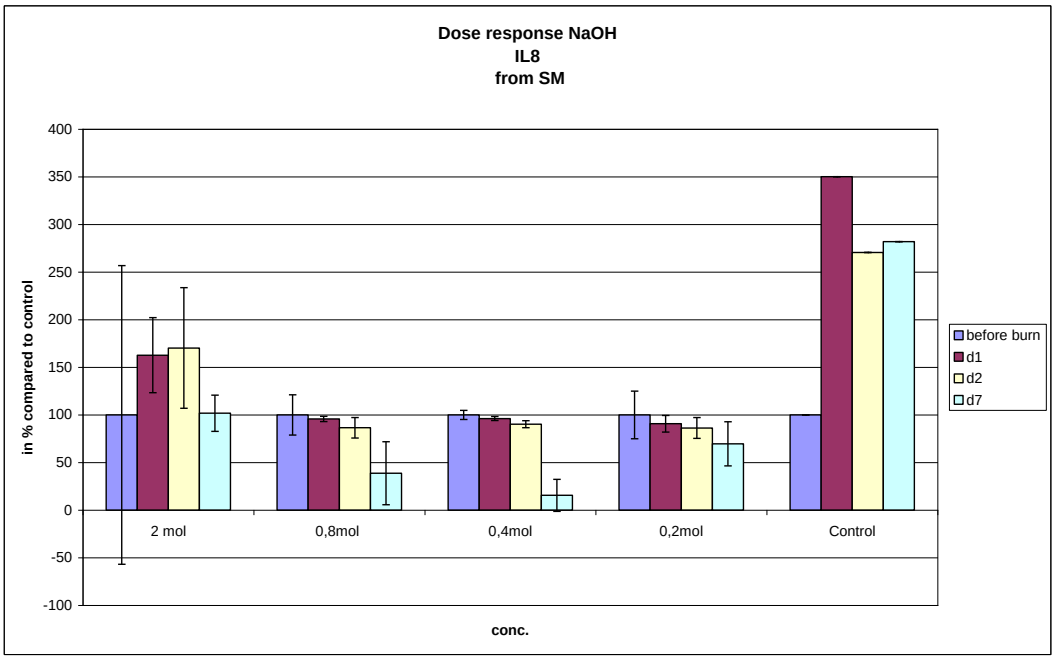

Fig. 73: IL-8 levels in the supernatant medium of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-8

In the supernatant rinsing medium the IL-8 was increased at six hours and on day two after exposure to 2.0 mol NaOH. With the other concentrations of NaOH a decrease of the IL-8 values over the days presented. The control group showed high boosts throughout the measurements after burn.

Dose response of IL-8 after H_2O_2 burn

Perfusion medium

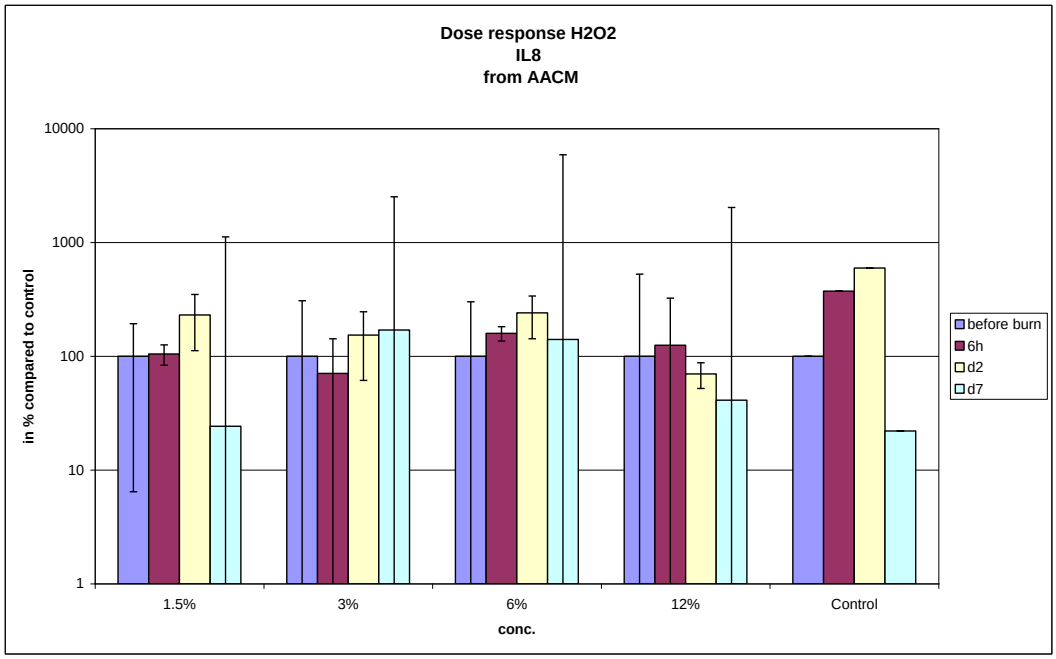

Fig. 74: IL-8 levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-8

After exposure of the surface of the ex vivo corneas to H_2O_2 in the perfusion medium the IL-8 levels were increased 2 days later: With 1.5% H_2O_2, by 100% of the initial values, and with 3% H_2O_2, by 50%. Irritation with 6% H_2O_2 resulted six hours after exposure in an increase of IL-8 by 50% and 2 days later by 100%. After exposure to 12.0% H_2O_2, the IL-8 levels were decreased

Supernatant medium

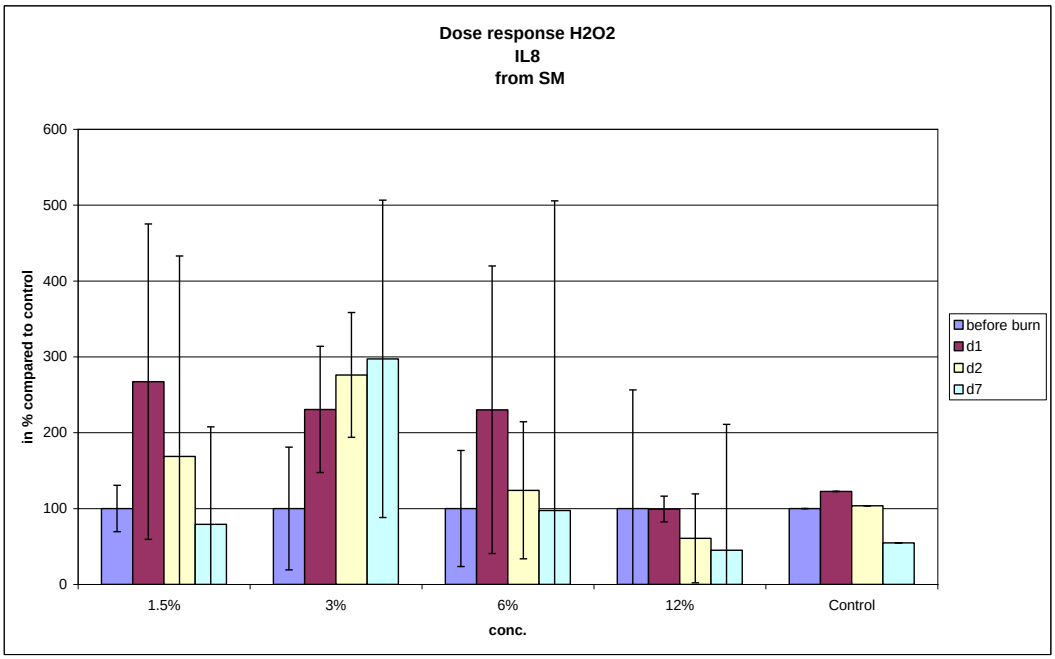

Fig. 75: IL-8 levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-8

The response on the surface of the ex vivo corneas, as presented in Fig. 75, to irritation by H_2O_2 was also very prominent. But the increase of the IL-8 levels in the supernatant rinsing medium appeared already on the first day after exposure. After exposure to 1.5% H_2O_2, the IL-8 levels were increased by 180% on day one, by 80% on day two. With 3.0% H_2O_2, IL-8 levels were elevated by 120% on day one, by 180% on day two, and by 200% on day seven. After exposure to 6.0% H_2O_2 the increase of IL-8 levels amounted 120% on day one only. After exposure to 12.0% H_2O_2, the IL-8 levels were decreased on days two and seven.

Dose response of IL-8 after TCA burn

Perfusion medium

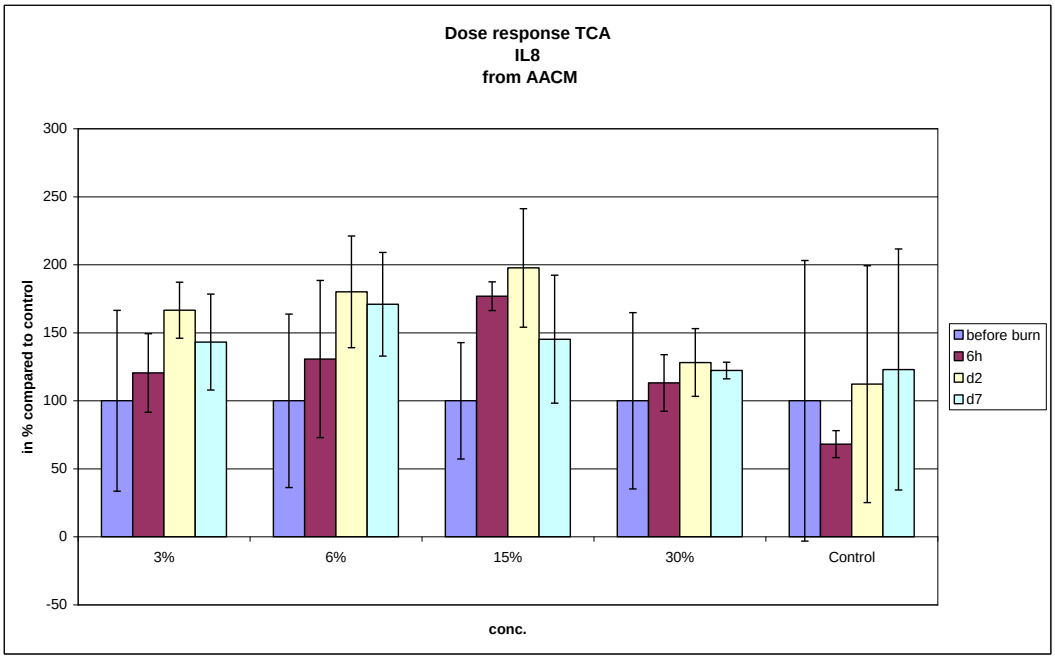

Fig. 76: IL-8 levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-8

Supernatant medium

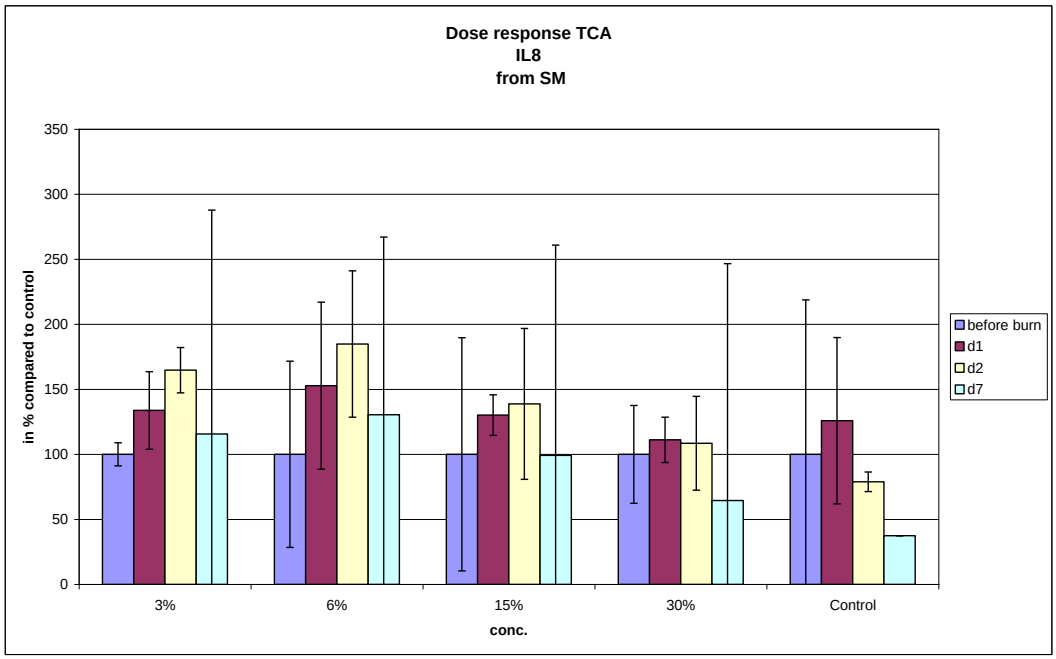

Fig. 77: IL-8 levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-8

The response of the ex vivo corneas to the exposure to trichloroacetic acid (TCA), was prominent in the perfusion medium and in the supernatant rinsing medium. After application of TCA to the cornea surface from the epithelial as well as from the endothelial side from day one to day two increasing amounts of IL-8 were released into both media (AACM and SM). Seven days after exposure, the IL-8 levels were decreased, but did not come back to the initial values. In detail, in the perfusion media (AACM), after exposure to 3%, 6%, and 15% TCA, the IL-8 levels were increased after six hours by 20%, 40%, and 80%, respectively; further on day two the IL-8 levels were increased by 60%, 80%, and 100% respectively of the initial values. The increase of IL-8 levels was more prominent in the AACM than in the SM. After exposure to 30% TCA only slight increases were observed, not consistent with the other TCA concentrations applied. In the supernatant rinsing medium the increase of IL-8 levels was much of the same as in the perfusion medium, but only after exposure to 3% and 6% TCA. With higher TCA concentrations applied, 15% and 30%, IL-8 levels were elevated only a little.

Dose response of IL-8 after SLS burn

Perfusion medium

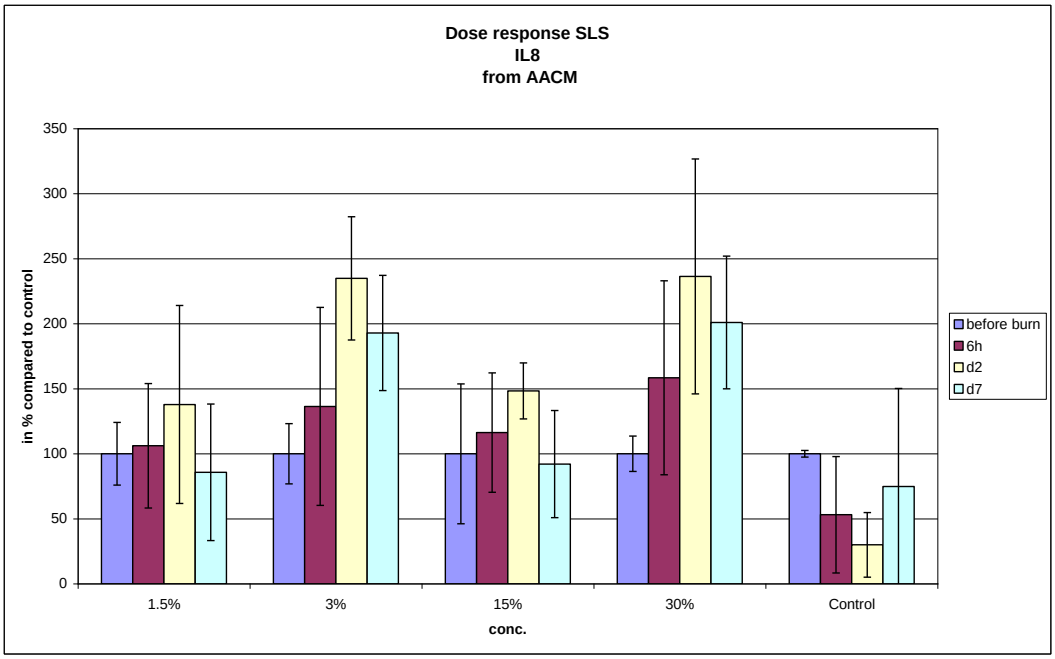

Fig. 78: IL-8 levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-8

Supernatant medium

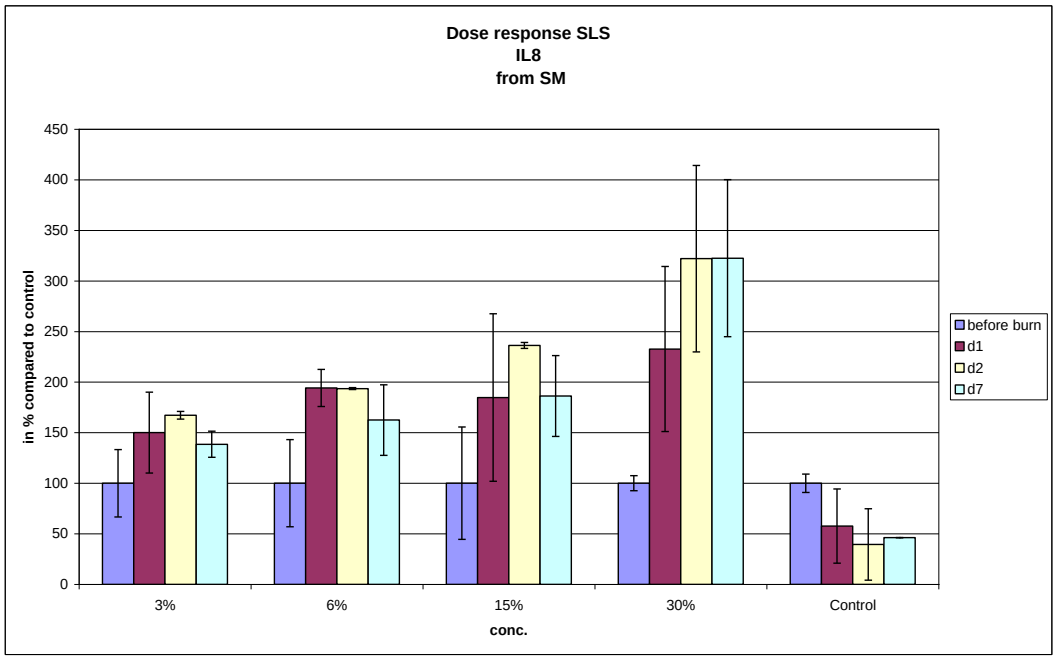

Fig. 79: IL-8 levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of IL-8

With exposure to sodium lauryl sulfate (SLS) the response by IL-8 release was much of the same as after TCA. But contrary to TCA after application of 30% SLS the IL-8 levels were still higher than after 3%, 6%, and 15% SLS. Not as much in the perfusion medium as in the supernatant rinsing medium the IL-8 increase seemed to be dose dependent with the day two values, which amounted with 3% SLS 60%; with 6% SLS 90%; with 15% SLS 140%; and with 30% SLS 220% of the initial values. The increase of IL-8 levels in the media on both sides of the ex vivo corneas increased with time and showed dose responses.

Dose response of MIP after NaOH burn

Perfusion medium

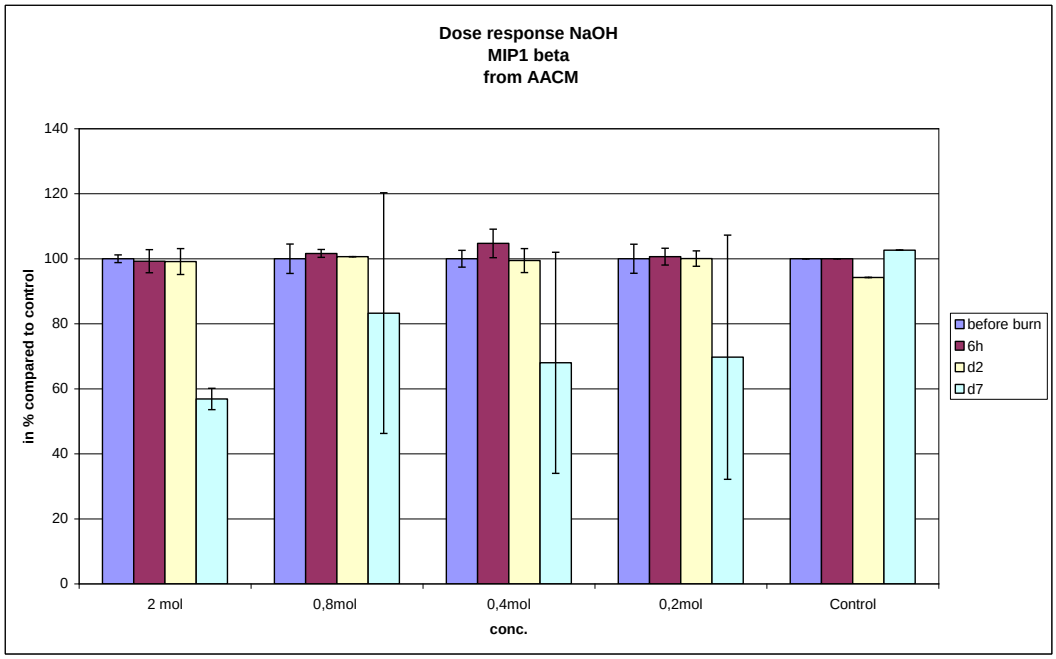

Fig. 80: MIP levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of MIP-1-beta

In the perfusion medium of ex vivo corneas MIP-1-beta was present at all times of the experiment. The exposure to various concentrations of NaOH did not change the MIP-1-beta levels in this medium initially, but seven days after the exposure to NaOH, the MIP-1-beta levels were decreased by 20% to 40% of the initial values.

Supernatant medium

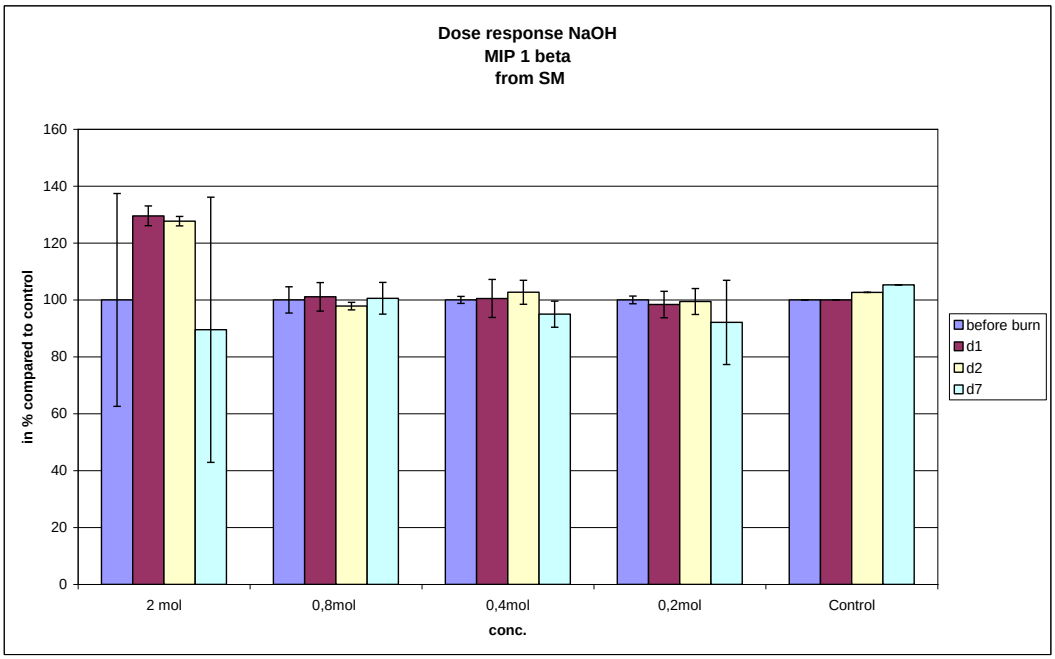

Fig. 81: MIP levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of MIP-1-beta

The MIP-1-beta levels in the supernatant rinsing media (SM), were increased by 30 % of the initial values only on days one and two after exposure to 2.0 mol NaOH, after exposure to the other concentrations of NaOH used in these experiments the MIP-1-beta remained nearly unchanged.

Dose response of MIP1-beta after H_2O_2 burn

Perfusion medium

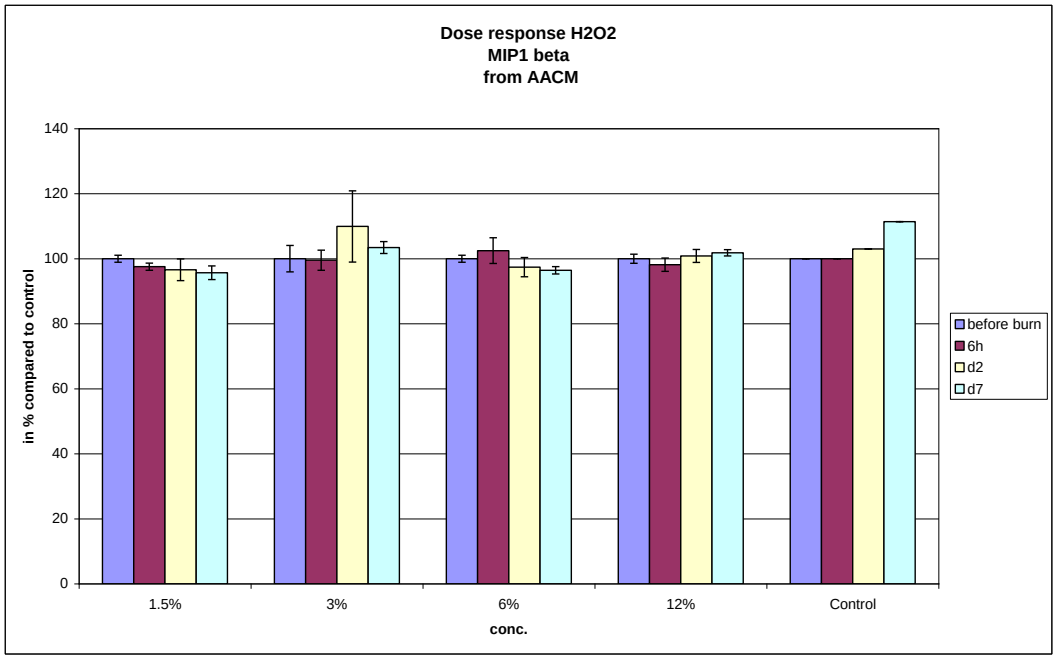

Fig. 82: MIP1-beta levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of MIP-1-beta

Supernatant medium

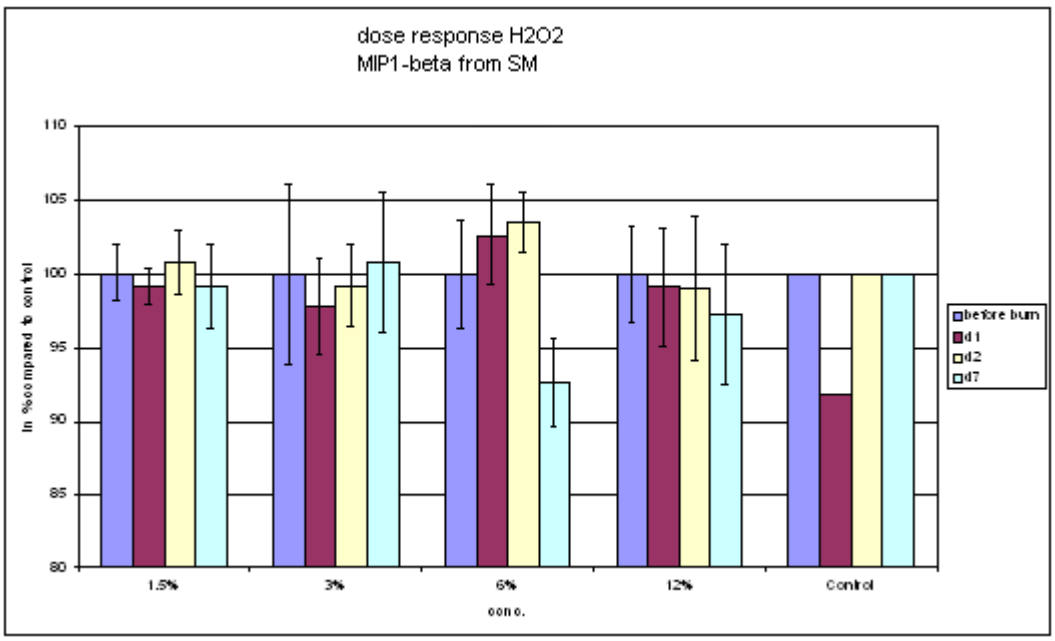

Fig. 83: MIP1-beta levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of MIP-1-beta

Like with NaOH exposure, with H_2O_2 no prominent change of the MIP-1-beta levels was observed in the perfusion and supernatant rinsing media over 7 days.

Dose response of MIP1-beta after TCA burn

Perfusion medium

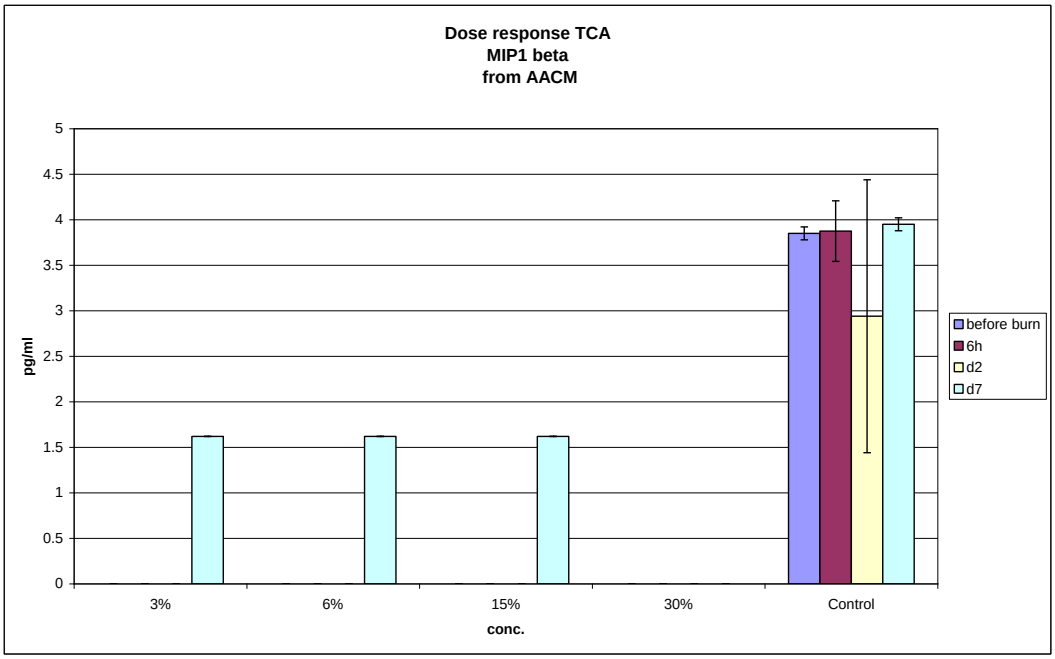

Fig. 84: MIP1-beta levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: amount of MIP-1-beta in pg/ml

Supernatant medium

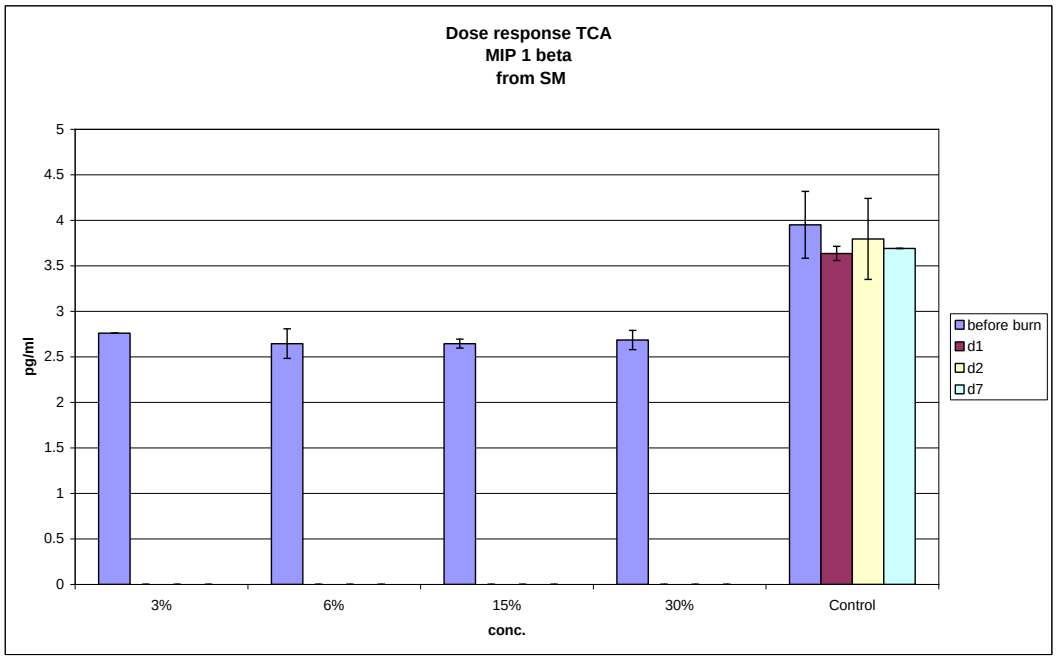

Fig. 85: MIP1-beta levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: amount of MIP-1-beta in pg/ml

After exposure of the ex vivo corneas to TCA, measurements of MIP-1-beta levels in the perfusion and supernatant rinsing media were rather difficult. Therefore, the results were given in absolute levels in pg/ml. The lower detection limit of the analyses was 2.12. pg/ml. As can be seen in diagrams of Fig. 84 and 85, in the exposure experiments, the values for the levels of MIP-1-beta were below the limit of detection in the perfusion medium, and slightly above in the supernatant rinsing medium. In the control corneas, however, the MIP-1-beta levels were well in reliable measuring ranges. Consequently, after exposure to TCA, MIP-1-beta disappeared from the media around the ex vivo cornea, as was observed with IL-1.

Dose response of MIP1-beta after SLS burn

Perfusion medium

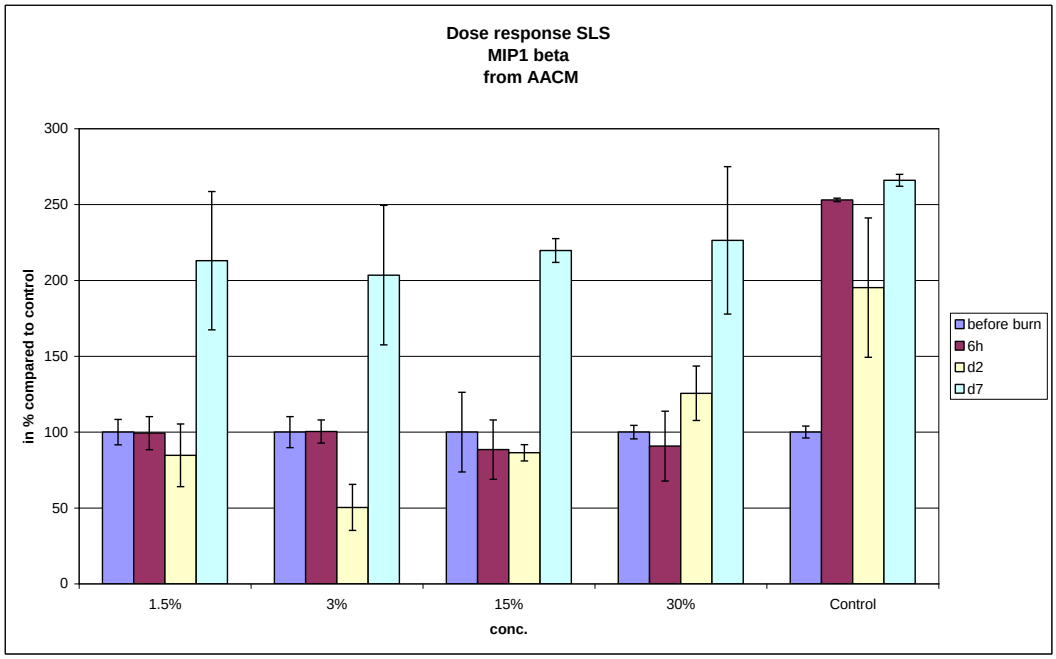

Fig. 86: MIP1-beta levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of MIP-1-beta

Supernatant medium

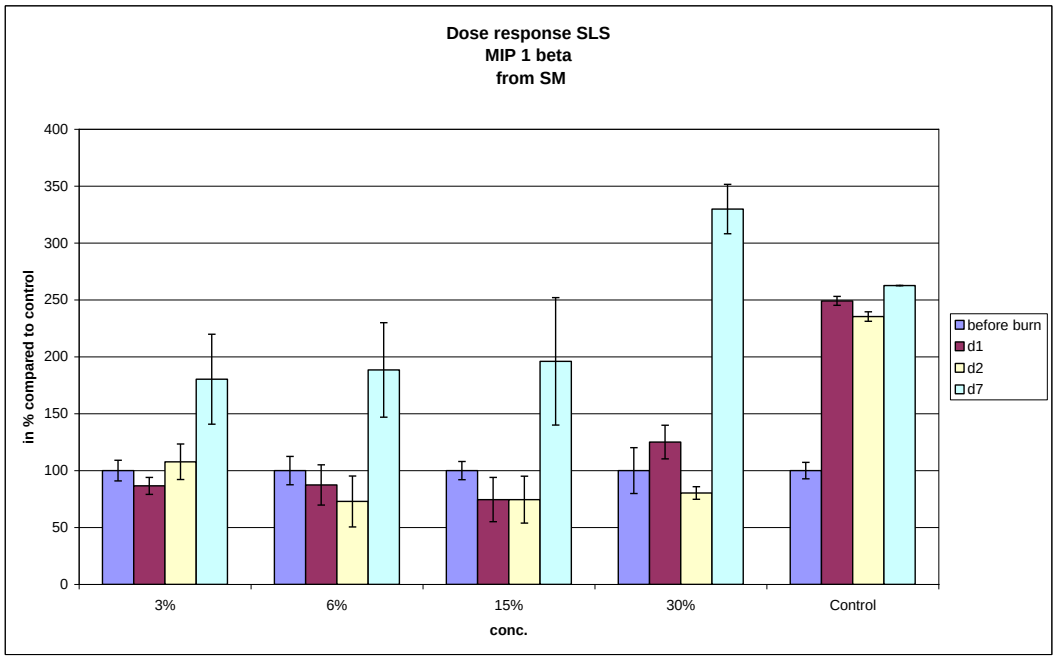

Fig. 87: MIP1-beta levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of MIP-1-beta

After exposure of the ex vivo corneas to sodium lauryl sulfate (SLS) MIP-1-beta levels in the perfusion and supernatant rinsing media were well established. There were some decreased levels of MIP-1-beta on days one and two, but the differences from the initial values were not significant. In both media, after exposure to various concentrations of SLS, the MIP-1-beta levels were increased not before seven days. Then the MIP-1-beta levels were increased by 100% in the perfusion media and by 150% to 200% in the supernatant rinsing medium. With SLS concentration of 30% the increase of MIP-1-beta was more than 200% of the initial values in the supernatant rinsing medium. Contrary to IL-1, after exposure to SLS, MIP-1-beta was released from the ex vivo corneas.

Dose response of VEGF after NaOH burn

Perfusion medium

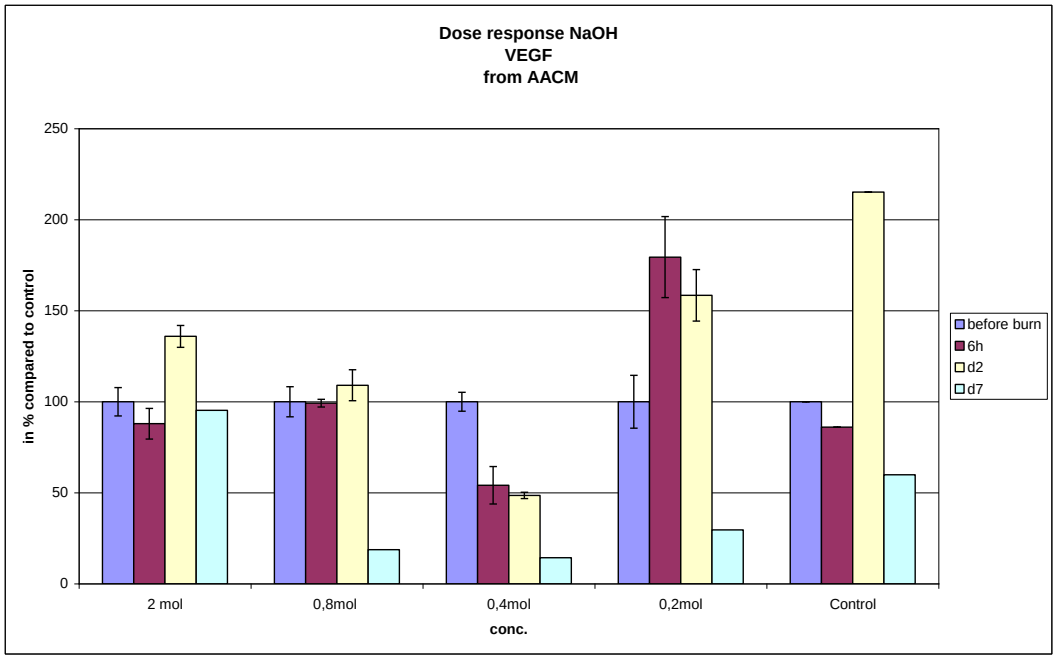

Fig. 88: VEGF levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of VEGF

After exposure of the surfaces of the ex vivo corneas to 0.2 mol NaOH in the perfusion medium the VEGF levels were increased by 50% of the initial values after six hours for two days. After seven days VEGF was decreased to 30% of the initial values. When the ex vivo corneas were exposed to 0.4 mol NaOH, after six hours and two days the VEGF levels were decreased to 50% of the initial values, after 7 days far more down to 15% of the initial values. After exposure to 0.8 mol NaOH, VEGF levels in the perfusion medium remained unchanged and decreased on day seven to 20% of the initial values. When 2.0 mol NaOH were applied to the surface of the ex vivo corneas, VEGF was slightly decreased after six hours and elevated by 40% of the initial values after 2 days. Seven days after exposure to 2.0 mol NaOH the VEGF levels were like the initial values.

Supernatant medium

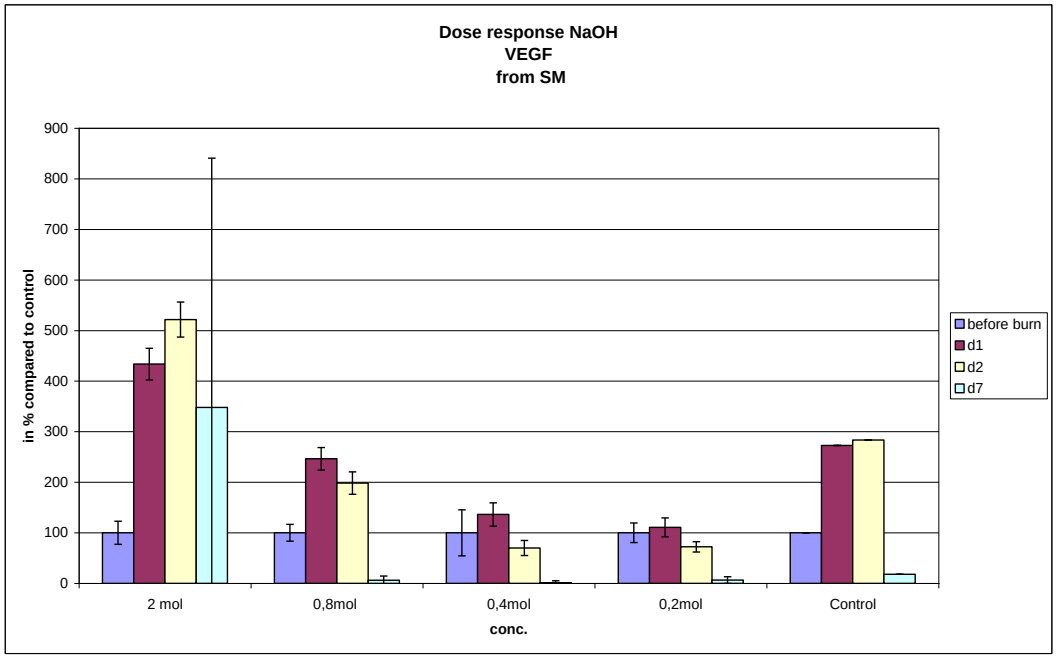

Fig. 89: VEGF levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of NaOH

Abscissa: various concentrations and time points; ordinate: procentual amount of VEGF

In the supernatant rinsing medium the VEGF levels changed in a different way than in the perfusion medium. After exposure of the surfaces of the ex vivo corneas to increasing concentrations of NaOH the VEGF levels were increased proportionally with rising NaOH concentrations. After exposure to 0.2 mol NaOH VEGF did not change for six hours, after two days was decreased to 80% of the initial values, and after seven days it was not found any more. After exposure to 0.4 mol NaOH six hours later VEGF was increased slightly by 20% of the initial values. Like after exposure to 0.2 mol NaOH after exposure to 0,4 mol NaOH in the supernatant rinsing medium VEGF levels were decreased on day two down to 60% of the initial values and after 7 days completely disappeared. When 0.8 mol NaOH were applied to the surface of the ex vivo corneas, after six hours, VEGF levels were increased by 120%, and after 2 days still by 100%, but after 7 days decreased to zero. Six hours after exposure to 2.0 mol NaOH in the supernatant rinsing medium VEGF levels were increased by 320%, after 2 days by 410% of the initial values and after 7 days VEGF levels were still elevated by 220% with an excessive high standard deviation.

Dose response of VEGF after H_2O_2 burn

Perfusion medium

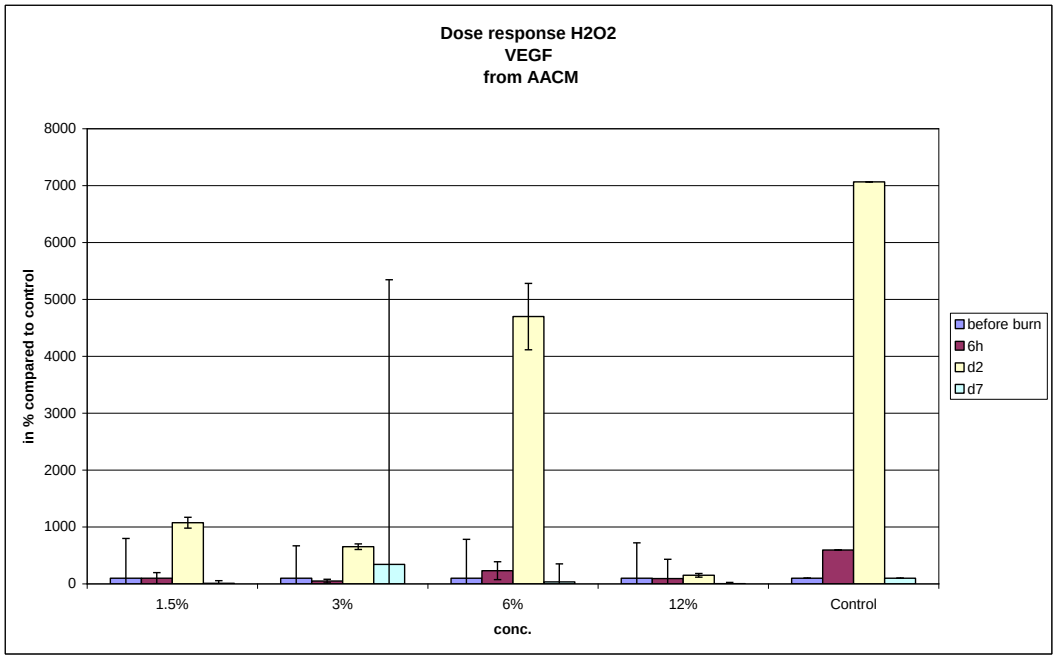

Fig. 90: VEGF levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of VEGF

Supernatant medium

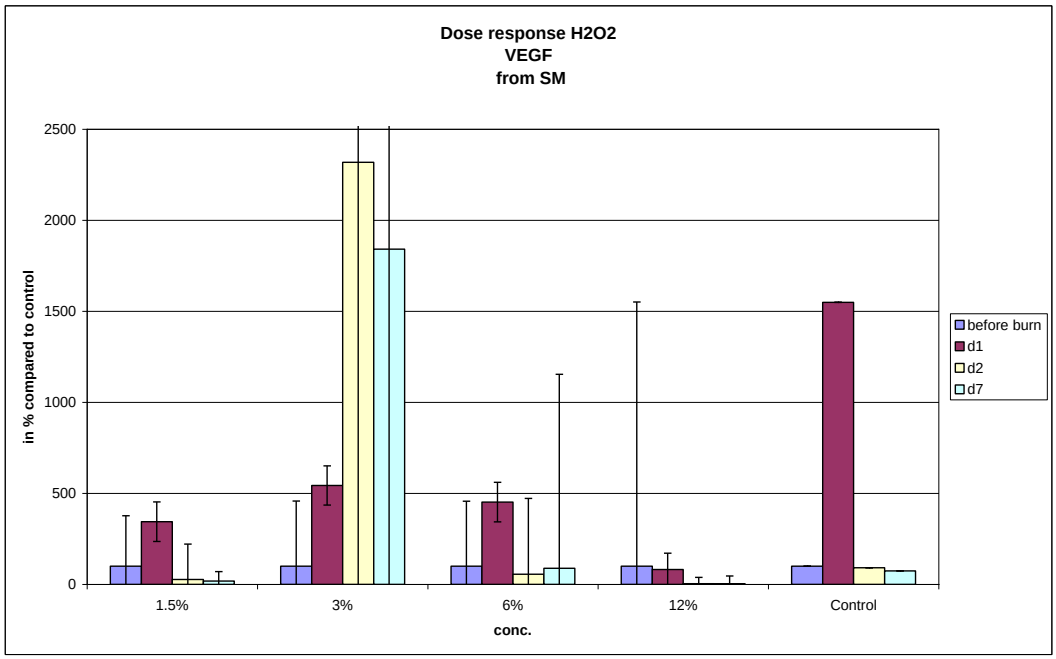

Fig. 91: VEGF levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of H_2O_2

Abscissa: various concentrations and time points; ordinate: procentual amount of VEGF

Exposure of the surfaces of the ex vivo corneas to H_2O_2 induced very prominent changes as excessive elevations of VEGF levels in the perfusion and the supernatant rinsing media where found. While no change of the VEGF levels was observed six hours after burn, two days after exposure to H_2O_2 VEGF levels in the perfusion medium were increased with 1.5% H_2O_2 by 1000%, i. e. 100 times the initial values. Seven days later, after exposure to 1.5% H_2O_2 the VEGF levels were decreased to less than 50% of the initial values. With exposure to 3% H_2O_2, the changes of VEGF levels induced in the perfusion medium were much the same. Six hours after exposure VEGF levels were less than the initial values, after two days elevated by 600% and after 7 days in the average by 300% with excessive high standard deviation. Six hours after exposure of the ex vivo corneas to 6% H_2O_2 VEGF was increased by 100%, after two days by 4500% and after seven days decreased to much less than the initial values. When 12% H_2O_2 were applied to the ex vivo corneas, no rise of VEGF levels in the perfusion medium was observed at all. Only after seven days VEGF was decreased to about 10% of the initial values.

Dose response of VEGF after TCA burn

Perfusion medium

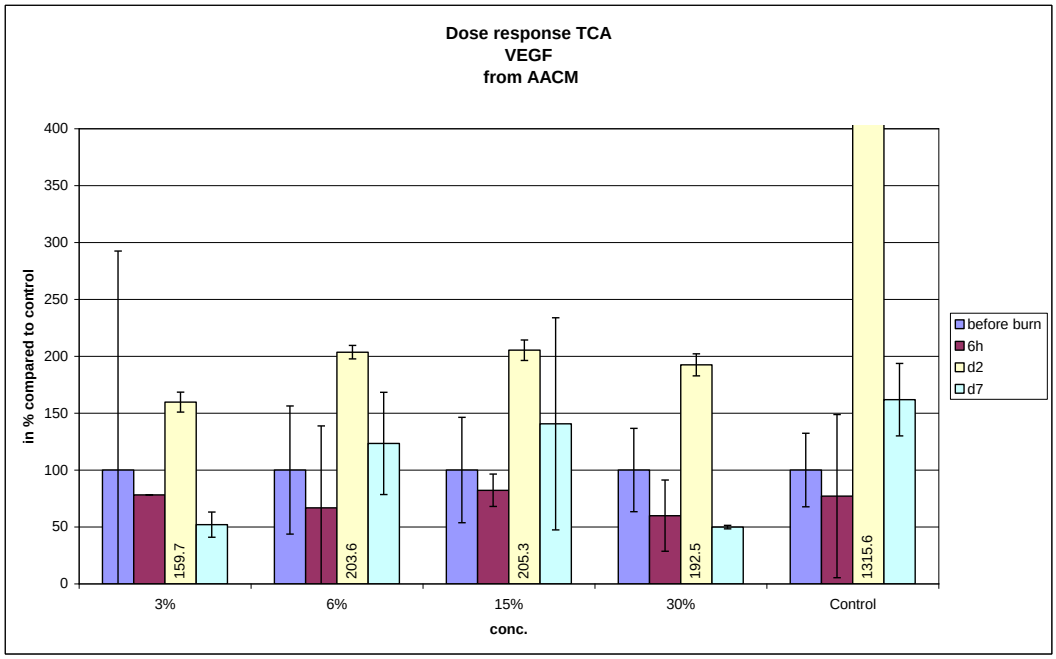

Fig. 92: VEGF levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: procentual amount of VEGF

In the perfusion medium six hours after exposure of the ex vivo corneas to trichloroacetic acid (TCA) with all concentrations of TCA applied the VEGF levels were decreased to 50% to 80% of the initial values. Two days after the exposure to 3%, 6%, 15%, and 30% TCA VEGF levels were increased by 50%, 100%, 100% and 90% of the initial values, respectively. Seven days after exposure, in the perfusion medium VEGF levels were decreased to 50% after 3% and 30% TCA, they were increased by 30% and 40% after 6% and 15% TCA. However, the changes of the control corneas were similar to the exposed ones, except that the VEGF of the unexposed cornea after two days went up by 1300%.

Supernatant medium

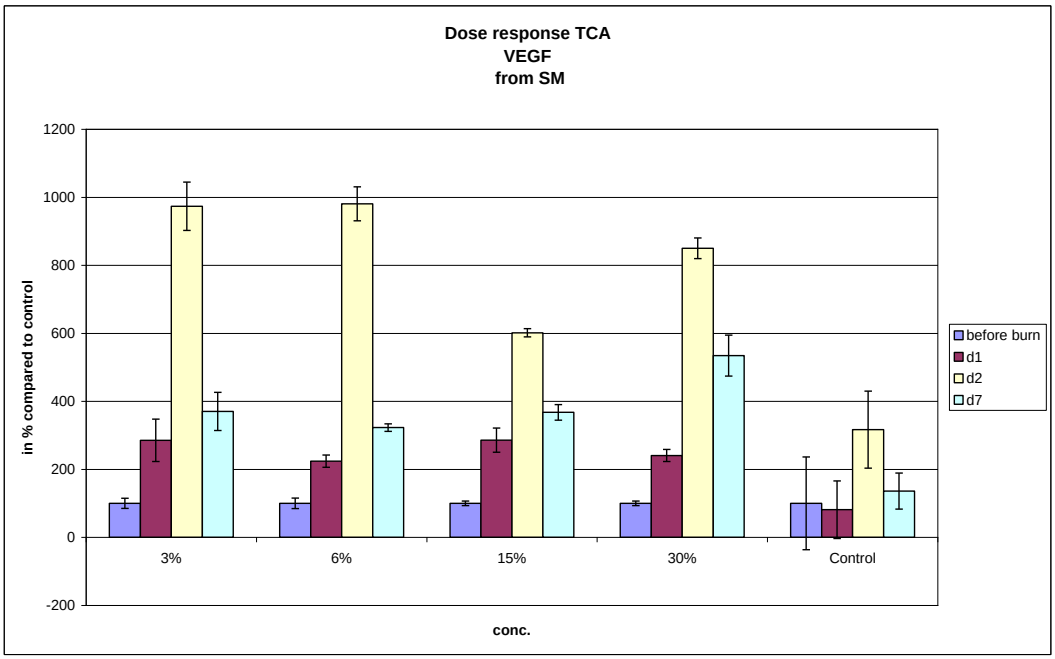

Fig. 93: VEGF levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of TCA

Abscissa: various concentrations and time points; ordinate: procentual amount of VEGF

In the supernatant rinsing medium after exposure to 3%, 6%, 15%, and 30% TCA VEGF levels showed a parallel, phasic increase after six hours by 100%, 60%, 80%, and 60%, respectively. Two days after erxposure to 3%, 6%, 15%, and 30% TCA maximum VEGF levels were increased by 880%, 880%, 500%, and 750% of the initial values. Control values behaved similarly, but with rather low levels of VEGF.

Dose response of VEGF after SLS burn

Perfusion medium

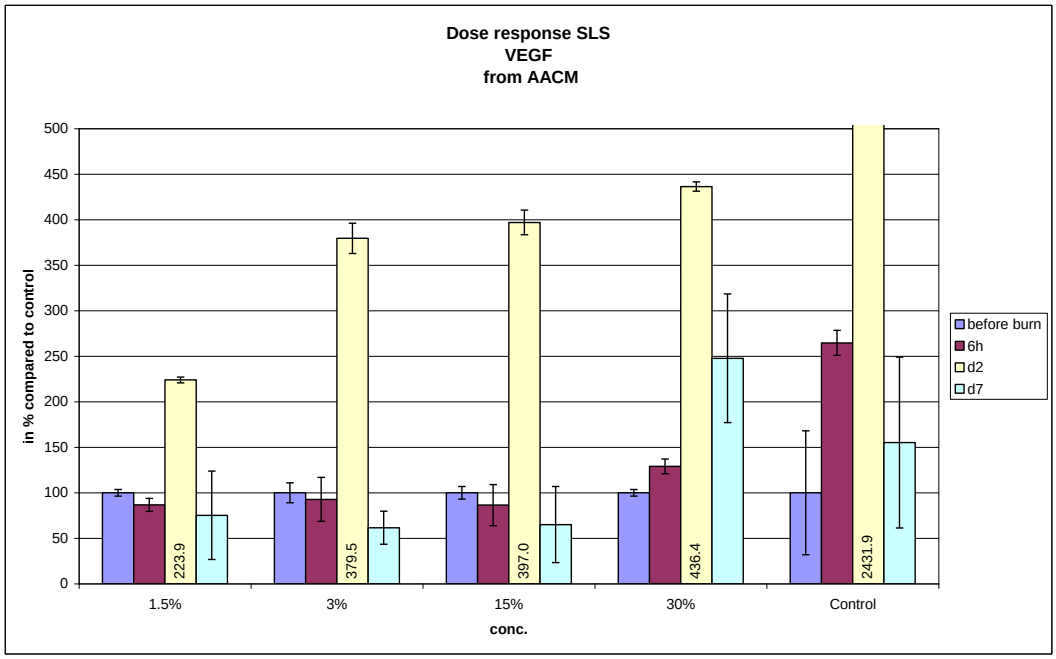

Fig. 94: VEGF levels in the perfusion medium (AACM) of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of VEGF

In the perfusion medium within six hours after exposure to various concentrations of sodium lauryl phosphate (SLS) VEFG levels did not change and were increased only slightly, by 30%, when 30% of SLS was applied. But two days after exposure to 1.5%, 3%, 15%, and 30% SLS VEGF levels in the perfusion medium were increased by 220%, 280%, 300%, and 350% of the initial values, respectively. The increase of VEGF levels in the perfusion medium after exposure to 1.5% and 3% SLS showed dose response. Seven days after exposure to 1.5%, 3%, and 15%, SLS VEGF levels were decreased to 60% to 70% below the initial values. But after 30% SLS exposure VEGF levels were still increased by 150% of the initial values. Again, like in the control corneas of TCA exposure, the control corneas to SLS experiments were very highly increased, after six hours by 150%, after two days by 2300% and after seven days by 50%.

Supernatant medium

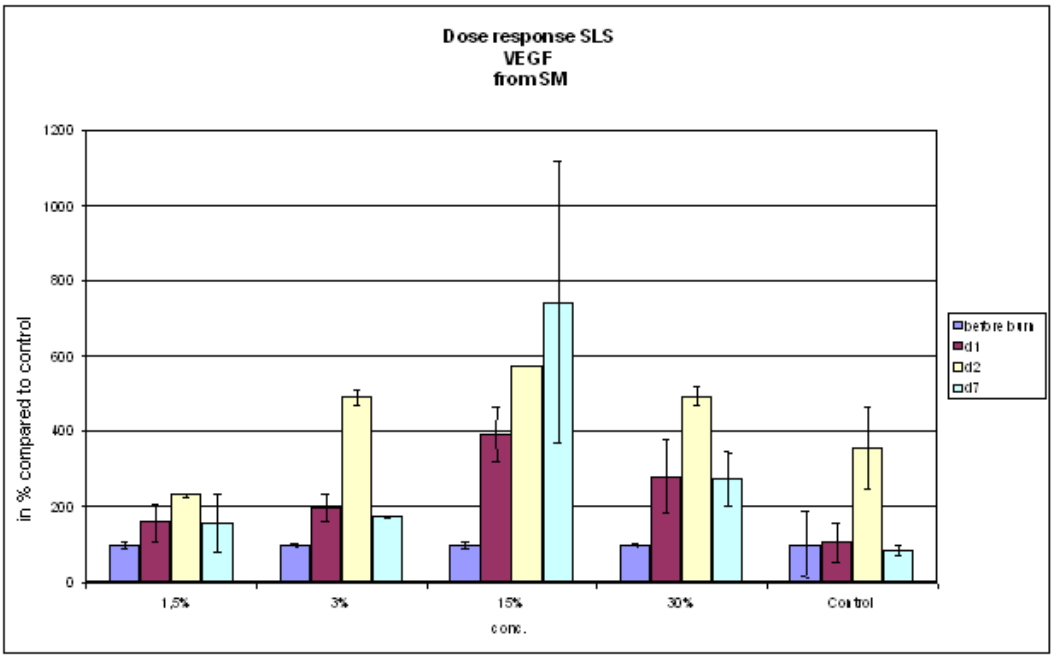

Fig. 95: VEGF levels in the supernatant medium (SM) of ex vivo corneas after exposure to various concentrations of SLS

Abscissa: various concentrations and time points; ordinate: procentual amount of VEGF

In the supernatant rinsing medium the exposure to 1.5%, 3%, 15%, and 30% SLS induced VEGF level increases in classical dose response functions with maximum values after exposure to 15% SLS on the time points investigated. On day 1 VEGF levels were increased by 60%, 100%, 300% and 200% after exposure to 1.5%, 3%, 15% and 30% SLS, respectively. On day two VEGF levels were increased by 110%, 400%, 600%, and 450% after exposure to 1.5%, 3%, 15%, and 30% SLS, respectively. On day seven VEGF levels were increased by 60%, 80%, 750% and 200% after exposure to 1.5%, 3%, 15%, and 30% SLS, respectively. The control corneas released VEGF only on day 2 by 250% above control values.

Discussion

MMAS and epithelial wound healing

The hardly increasing MMAS scores of the pictures taken of the untreated corneas show that apart from cell detritus that accumulated on the corneas' surfaces nearly no epithelial damage could be detected after culturing these corneas in the EVEIT system. Concerning morphological criteria the EVEIT seems thus to be able to keep alive cultured corneas. Furthermore, as fig. 9 and 10 show after abrasions done on the corneas nearly complete epithelial wound healing could be demonstrated after about five days. As corneas treated with H_2O_2 3%, NaOH 2mol, tomadol 47-5 3% and tomadol 14-5 10% however showed either only a slight healing of the initial epithelial defect or even no healing resp. enlargement of the primary defects (see fig. 9 and 10) it is obvious that by means of the EVEIT system the potential of chemicals to cause damage on corneas can be quantified. To search out if there is any dose dependent effect we performed experiments with different concentrations of the same chemicals. Though some difficulties appeared:

We performed the testings wih TCA in the concentrations of 3%, 6%, 15%, and 30%. TCA is frequently used for biochemical analysis; complete precipitation of tissue protein is then achieved with 6% TCA. Since such TCA concentrations are applied in analytic techniques since more than hundred years, it may be allowed, to assume, that in the experiments of fig. 13, maximum toxicity of TCA may have been surpassed, so that a clear dose dependence can not be expected when using these high concentrations as done by us. Dose responses on TCA-application may be found in concentrations far below 6%.

The testings with SLS did not show any dose dependence. In the groups of 1,5%, 3% and 15% concentration nearly equal MMAS scores were found. It is noticeable that corneas burnt with 30% SLS showed much lower MMAS. This could have been due to the rather high viscosity of 30 % SLS, so that it might not have been transferred properly to the surface of the ex vivo cornea from the contact lens used for application in these experiments.

The apparent, but not significantly less severe MMAS values after exposure to 2mol NaOH might have been due to a different application technique in these experiments, so that a comparision of the MMAS may not necessarily be allowed.

Altogether the bars for the higher concentrations in the diagrams fig. 12-14 on the right sides suggested to conclude, that the concentrations of TCA, SLS and H_2O_2 selected for investigation with the ex vivo cornea system were at maximum toxicity and therefore too high to reveal dose responses. When having a look at the MMAS scores found in these experiments nearly the same phenomenen can be detected: Especially the tests with TCA propose that the used concentrations were too high to obtain dose responses. Consequently, further experiments may be expedient to search for dose responses with lower exposure concentrations. Also higher numbers of experiments per concentration group may reveal significant dose responses. However, the absolute mean values of differences between concentration groups should be large and the standard deviations small enough to separate single measurements from average comparators.

Endothelial damage

The endothelial damage after burn with NaOH on day 7 shows a clear dose response, which may become statistically significant with a larger number of cases. These data support the assumption known from multiple experiments, that alkali penetrates the corneal stroma in a measurable time, which is longer with lower concentrations of NaOH, such as 0.2 or 0.4 mol and which becomes much shorter in strong alkali burns (Spoler, Forst et al. 2007). The time course of the increasing endothelial damage also shows the need for not only immediate, but also extended irrigation of alkali burnt eyes.
The nearly non existent endothelial damage immediately after burn with H_2O_2 with either concentration shows, that H_2O_2 penetrates slowly through the corneal stroma. It is not until the second day that remarkable endothelial affection can be detected.
The low effect of TCA concerning endothelial damage as shown in fig. 17 is well known from its use for precipitation of proteins in analytical biochemistry, and from peeling therapy of the skin with TCA where a concentration dependent clear cut chemical debridement of the skin can be done leaving out the germinative zones of the skin (Fulton and Porumb 2004). So it is not surprising that in our testings no intense effect on the endothelium could be demonstrated.
The results of the experiments witht SLS are not that significant. As mentioned above the high viscosity of 30% SLS may have caused difficulties concerning the potential of penetrating the cornea and the follwing effects on the endothelium. Nevertheless, endo-

thelial damage was achieved by SLS, but the little numbers of experiments do not allow any further evaluation.

pH and Glucose/lactate measurements in single dose experiments

In the experiments with unexposed ex vivo corneas it was evident that the test system performed constant values for pH, glucose and lactate levels on both surfaces of the cornea, the endothelium as well as the epithelium over 9 days. As there is no lactate in the perfusion medium, any detected lactate contents must be considered as being produced by the corneas. While the pH simply showed physiological equilibrium of tissue fluids, the glucose and lactate levels represented active glucose metabolism, which is an indicator for survival of the corneas in the EVEIT system.

The pH of the outflowing medium increased when larger defects of the corneal epithelium occurred. The reason for this pH-elevation may be seen in the loss of carbon dioxide through the denuded surface of the corneal stroma. Severe irritation, such as mechanical abrasion, chemical injuries from tomadol E 14-5, H_2O_2 and NaOH produced important and reproducible disturbances of the glucose metabolism. It may be concluded, that the change of the glucose and lactate levels was caused by the mechanical trauma of abrasion or burn and the transitory loss of large parts of the corneal epithelium. Even more important than the break-in of the metabolism was the observation of its enduring revival. This should be interpreted in a sense, that the chemical traumata did not produce complete cell death or necrosis and moreover that the corneas within this test system are able to recover from damage. Biochemical signs of survival of cell metabolism should be distinguished from damage to specific tissues. And this was found in the extensive morphological investigations of the same ex vivo corneas. Epithelial defects, corneal turbidity or even corneal opacity and endothelial damage were evident. But these morphological changes could be graded and then became useful tools to measure toxicity.

In the biochemical analyses of the perfusion media as well as in the morphological findings, the damages and responses of the ex vivo corneas to the various chemicals were different. From these results it is possible to define specific damage patterns for the chemicals investigated by epithelial regeneration, stromal opacifications, endothelial damage and biochemical changes. All of these parameters can be observed over time, i.e. in the development of the damage and in recovery.

pH and Glucose/lactate measurements in dose response testings

Glucose and Lactate monitoring showed that the system itself is stable and even under exposition with different chemicals still alive. The lactate levels decreased the more the corneas became opaque, so that there is a direct correlation between these two criteria of metabolism and morphology. Over and above the glucose and lactate monitoring was used as an entrance criterium to sort out these corneas which were not viable enough for ex vivo testing. All corneas monitored for dose response analysis fulfilled the criteria to produce in the perfusion medium more than 1.0 mmol lactate before burn. No further documentation is reported here, if this entrance criterium was missing. After exposure to higher concentrations of H_2O_2 and NaOH, the lactate levels in the perfusion media decreased in a significant way, remaining high after exposure to TCA and SLS on the other hand indicating less damage to glucose turnover in these corneas. After exposure to H_2O_2 and NaOH, in the supernatant rinsing media, lactate levels were low and consistent with a decrease of glucose turnover. In contrast, after SLS and TCA exposure, in the end of the experiment, the lactate production found in the in the supernatant rinsing media returned to the starting values indicating a biochemical recovery of the anterior stroma and of survived epithelium.

Comparing the results of the dose response studies, it becomes apparent, that each of the chemicals selected to test the irritation of the ex vivo corneas produced an individual pattern of damage, not only in morphological but also in biochemical investigations. In the perfusion media, the response to the alkali NaOH induced disturbance of the glucose consumption with a latency of one to two days, the response to H_2O_2, feasible in the perfusion media, began immediately after the exposure. Dose responses were found with NaOH and H_2O_2, especially with regard to the decrease of lactate levels. TCA and SLS did not impair the glucose metabolism as much, TCA likely because of the precipitation of protein, SLS because of its high viscosity. Presumably, both of these effects inhibited the expansion of the chemical damage. However, unlike to the first experiments reported in Fig. 23, 25, 27, 29 and 31 recovery of lactate and glucose levels was not found in the dose response experiments demonstrated in Fig. 33 to 36. It has to be explored, whether this deviation from previous results was due to details of the experimental conditions. Especially the pH variations and the buffering capacity of the medium seem to be of higher importance than previously observed. In later experiments with the system we gave proof of this fact and we believe that the stability of the ph within the medium makes a huge difference in results.

Cytokines and growth factors

Cytokines were found in nearly every sample. In some experiments, prominent changes were found, which differed markedly from normal ranges of control series. There were evidently specific responses of the ex vivo rabbit corneas to exposure of distinct chemicals. The results of the measurements of IL1, e.g. increased levels after burn with NaOH and H_2O_2, are compatible with cellular response to chemical trauma. The lower levels from Tomadol E14-5, hardly exceeding the lower limit of detection only at 8 hours and one day after exposure might indicate extended necrosis due to Tomadol E14-5**.** The findings with IL-8 levels in the perfusion media from ex vivo corneas after exposure to H_2O_2 and NaOH were contrary to the ones with IL-1. These results lead to the conclusion, that in the ex vivo corneas IL-1 and IL-8 showed different responses to H_2O_2 and NaOH, which may be specific for the damage by these chemicals. An increase of MIP-1-beta could be observed in all of our testings as well as in LPS stimulated rabbit blood cells. As a macrophage inhibiting factor, MIP-1-beta, is essential for tissue responses. Since it was found also in the perfusion medium from corneas "without trauma", the preparation procedure seemed to be sufficiently traumatic to produce MIP-1-beta in the controls. FGF is involved in the induction of tissue proliferation and release of matrix-metalloproteinases, and therefore, is associated with corneal ulceration. The very high FGF levels immediately after preparation of the ex vivo corneas might have been caused by the preparation trauma. Vice versa, the low levels or just the absence of FGF during the whole time of the experiment suggested, that the ex vivo system was stable. The results regarding the VEGF levels after NaOH-burn, which are rather low in comparison with these after burn with H_2O_2 are remarkable, but have to be reproduced, since clinically burns with NaOH clearly induce corneal vascularisation. However, up to now it is not known, whether after alkali burns, corneal vascularisation is induced from the adjacent conjunctiva or from the cornea itself. The ex vivo cornea system may be apt to decide this question. After exposure to tomadol E14-5, VEGF-165 exceeded generally initial values, but not the levels of corneas without trauma, suggesting that tomadol E14-5 did not induce a specific VEGF-165 response of the cornea.

Dose response of FGF

The FGF levels in the perfusion medium after burn with different concentrations of NaOH did not show much alteration except at the time point 6 hours after burn with the

concentration of 2mmol. However, standard deviations were excessively high. Therefore, interpretation of this result should be done very reluctant. While 1.5% H_2O_2 may have been a sub threshold irritation it may be concluded that with a dose of 3% H_2O_2 the response of the ex vivo corneas was very prominent and lasted up to 7 days. On higher H_2O_2 concentrations, in view to the low FGF release from the control corneas, it may be speculated, that the damage to exposed corneas may have been repaired so far that no FGF was released any more. However, the severe damage to the corneal surface and to the corneal endothelium as seen in Fig. 12 and 16, respectively, showing increasing morphological damage with time does not correspond to healing processes. Therefore, the low FGF release from the ex vivo corneas on the second and seventh days after exposure to H_2O_2, is more consistent with cell damage than recovery. Then the decreased FGF in the controls remains to be explained. The results with exposure to TCA are remarkable, because many data were missing, although technical and experimental problems could not be identified. If the single high value on 6 hours after 30% TCA exposure is not considered, the fact that no FGF was found neither in the perfusion media nor in the supernatant rinsing media after exposure to TCA cannot be neglected. Remembering, that TCA precipitates proteins completely even with 3% concentration, this result corresponds with the results of the morphological results presented in Fig 13 and 17 that TCA destroys cells and consequently extinguishes all cellular responses. In the experiments with 1.5% SLS a short but prominent release of FGF was observed. But with higher concentrations of SLS the FGF decreased. These results were not found after exposure to NaOH as clearly as with H_2O_2, TCA and SLS. With exposure to NaOH the maximum response of FGF was probably seen with 0.4 mol NaOH. But the penetration characteristic of the alkali is different from H_2O_2, TCA and SLS in that NaOH diffuses not only perpendicular through the cornea, but also expands in lateral direction. Consequently as observed in alkali burns with clinical patients marginal cornea, not directly touched with the exposure, are expected to have been damaged but not destroyed. Therefore, regeneration processes or inflammatory responses of surviving cells may have produced more FGF than with damage from H_2O_2, TCA and SLS. Another observation in the FGF analyses leads to the conclusion, that with the concentrations of chemicals applied in most experiments a maximum damage was set to the ex vivo corneas, which presumably for that reason could not develop recovery and cellular responses.

In the supernatant rinsing media from exposed ex vivo corneas (SM) the FGF levels showed very high variation in the control as well as in the exposure experiments. It seemed that in the present state of the ex vivo cornea system, accidental surface alterations, mainly of the corneal epithelium, occurred even in untouched normal corneas and apparently induced FGF responses which on the first view could not be explained. But the unexpected very high FGF release in the controls to the NaOH and the SLS exposure (Fig. 48 and 54), reminded of small surface defects as discussed with epithelial structures observed in the untreated ex vivo corneas of Fig. 9 and 10. Otherwise the FGF release from the corneal surface to the surface rinsing media resembled the results obtained from perfusion media.

The differences of these responses to chemical injuries should be investigated further to allow for more valid interpretation.

Dose response of IL-1-alpha

After exposure of the corneal surface to 0.2 and 0.4 mol NaOH a release of IL-1-alpha to the perfusion medium, i. e. on the endothelial side of the cornea, was evident. But on exposure to 0.8 and 2.0 mol NaOH nearly no release of IL-1-alpha to the perfusion medium could be detected. Presumably, the amount of destroyed tissue may have been that large that production of IL-1-alpha ceased. In the perfusion medium of the H_2O_2 burned corneas (1.5%, 3% and 6%) only on the third day slightly increased IL-1-alpha values were obvious. No release was measured after burn with H_2O_2 12% at no time point. The same explanation as for the lack of IL-1-alpha after burn with higher concentration with NaOH may be appropriate. After exposure to TCA no IL-1-alpha could be measured neither in the perfusion nor the supernatant rinsing media. The results were similar to those found in the FGF analyses, likely because TCA precipitated all proteins and consequently eliminated almost all cellular responses.

Considering the results of the slitlamp microscopical examination of the ex vivo corneas as demonstrated in Fig. 14 and 18 (epithelial and endothelial healing/damage after SLS-exposure) there was evidence of rather severe morphological damage to the epithelium with increasing damage score from 1 to 3 till to the seventh day and moderate damage to the endothelium reaching a damage score of 1.0 to 1.5 after seven days following the exposure. The lack of release of IL-1-alpha after SLS exposure from both sides of the ex vivo cornea into the perfusion and the supernatant rinsing media during the first two days after exposure and rather high levels of IL-1-alpha in both of the media after 7

days is difficult to be explained, maybe it reflects some recovery of cells in the later stages of the experiment, which would not be consistent with the morphological results as mentioned above. But technical failures were not recorded so that SLS may have suppressed cell functions and thereby suppressed the synthesis and/or release of IL-1-alpha and, as mentioned in the next section, IL-1-beta into the perfusion and supernatant rinsing media. But cell structures may not have been destroyed permanently by SLS. Therefore when after some time from exposure SLS was diluted and rinsed out from the tissues cell functions recovered and IL-1-alpha and IL-1-beta synthesis were resumed and these cytokines reappeared in the media on day 7. However, this interpretation does not fit to the prominent response with high and permanent release of IL-8 after exposure to TCA and SLS, which will be reported in the accordant section.

Dose response IL-1-beta

The increase of IL-1-beta in the perfusion medium 6 hours after exposure on NaOH 0.4 and 0.8mol may have been related to the specific effect of moderate chemical alkali trauma by these concentrations of NaOH. The severe chemical burn by 2 mol NaOH apparently destroyed a large amount of corneal tissue that was able to produce cytokines, and consequently reduced the production and release of IL-1-beta into the perfusion media. By continued and expanding chemical trauma, which is typical for alkali burns, this mechanism might have further reduced the release of IL-1-beta in the later stages of the experiment on days 2 and 7. After 6% and 12% H_2O_2 exposure, some higher release of IL-1 beta was observed on day 2 in the AACM and the SM media. This observation indicated later onset of damage by H_2O_2 which penetrates more slowly through tissues compared to NaOH. Also the damage observed in the endothelium of the ex vivo corneas after exposure to H_2O_2 as demonstrated in Fig. 16, developed slowly reaching its maximum not before day 7. But since one can detect a clear increase in IL-1-beta in H_2O_2 6% and 12% burned corneas as well as in the control group both on the second day it can not be excluded that this prominent IL-1-beta release could have been an artifact or an unspecific response, possibly due to superficial epithel irregularities. As the supernatant rinsing medium (SM) showed also very high levels of IL-1-beta after exposure to 3% and 6% H_2O_2 on day two IL-1-beta release by the ex vivo cornea exposed to H_2O_2 may have nevertheless some specific pathological significance. But the experiment should be reproduced to rule out artifacts by systematic experimental mis-

takes. Like in the analyses of FGF and IL-1-alpha after exposure to TCA, no IL-1-alpha was detected in the perfusion and supernatant rinsing media. The results are consistent with the fact, that TCA precipitated all proteins, and consequently eliminated almost all cellular responses. The results of the IL-1-beta release after SLS burn show, as mentioned above, the same pattern as the ones of IL-1-alpha.

IL 8 dose response

Six hours after exposure to NaOH in the perfusion medium the levels of IL-8 increased not very much but in line with decreasing NaOH concentrations from 2 mol to 0.2 mol NaOH, so with exposure to NaOH there was a rather clear inverse dose dependent increase of IL-8 levels in the perfusion medium but not in the supernatant rinsing media where the IL-8 levels six hours after exposure were elevated only with 2 mol NaOH but decreased with lower NaOH concentrations. The changes of the IL-8 levels in the perfusion medium (AACM) after exposure to NaOH did not differ very much from the findings in the other cytokines reported before. Like the other cytokines investigated, IL-8 levels decreased with time from day one to day seven in the perfusion medium as well as in the supernatant rinsing medium. This behaviour seemed to be a common feature after NaOH irritation, as one can see at the testings with other measured interleukins as well. After application of H_2O_2 1.5%, 3% and 6% the IL-8 levels in the perfusion medium increased considerably two days after exposure, consecutively remained constant or decreased. In the supernatant rinsing media, IL-8 levels increased also very much with lower, but decreased with higher H_2O_2 concentrations. This increase was extended to day 2 with 1.5% and to day 7 with 3.0% H_2O_2. With 6% H_2O_2, IL-8 was elevated only on the first day after exposure. After TCA as well as after SLS exposure, the ex vivo cornea responded with release of rather large amounts of IL-8. This behavior was very much different from an almost complete lack of release of IL-1-alpha and IL-1-beta into the perfusion and supernatant rinsing media (Fig. 59 - 62 and 67 - 70). But the IL-8 levels showed rather consistently increasing values with time and also with the TCA and SLS concentrations. As these analyses were performed from the same experiments and the same media samples, the difference in behavior of IL-1 and IL-8 has to be accepted, although cannot be explained satisfactorily by now. But a repetition of these experiments and analyses is advisable.

Six hours after exposure to NaOH, there was a tendency of increased levels in the perfu-

sion media, for FGF with 2 mol NaOH for IL-1-alpha and IL-8 with 0.4 and 0.2 mol NaOH, and for IL-1-beta with 0.4 and 0.8 mol NaOH. With NaOH concentrations in the borderline between mild and severe chemical trauma by NaOH, i. e. about at 0.4 mol NaOH, the interleukins investigated, IL-1 and IL-8, were preferably released into the perfusion media. FGF was released more prominently after exposure to strong alkali, 2.0 mol NaOH.

MIP dose response

The distribution of MIP 1 after burn with NaOH and H_2O_2 each in different concentrations seems not to be very significant. The distinct low levels after NaOH burn at day seven can be caused by severe tissue detruction.

VEGF dose response

No dose dependency in the experiments with NaOH could be detected, and the pattern of the VEGF values itself was very inconsistent as there are in- and decreasese in the values during the days ith different concecntrations of NaOH used without any clear paradigm, so that any specs are counterproductive. The discrepancy in the release of cytokines after TCA exposure is difficult to explain.One can speculate if it is perhaps a specific chemical reaction in the cornea that leads to such excessive production of VEGF or if it is the indulged precipitation of proteins by TCA that makes all MIP, IL-1 alpha and FGF disappear from perfusion media.
Which role did play the experimental technique of exposure, that possibly a limited portion of the ex vivo cornea was touched by the chemicals? And what role plays the surviving marginal corneal epithelium and stroma, under these conditions?
What kind of artifacts can happen at all in the experiments with the present system of ex vivo corneas?

The present results, collected in this volume, are worth to be completed and, where inconsistent, to be confirmed.

Comments on statistical mediator correlation analyses matrices

By means of statistics highly significant correlations are found that in view of rather low coefficients were not expected to be so close as statistics might give. This is mostly

related to the fact that the correlation coefficient (r^2) exceeds hardly 0.4. Nevertheless the positive correlations found in this small study give hope that dose response patterns can be detected in larger follow up studies with homogenous testing on larger groups exceeding the number of 3 corneas per concentration which is considered too weak for statistical testing.

In the data set represented graphically in the diagrams of the preceding chapters, dose responses were found if the starting points of individual experiments were set to 100% at the pre-incubation time of 36 hours. The biological problem of the interpretation of research on dose responses includes two problems: Synthesis and liberation of pre-formed mediators. In early time up to 6 hours after exposure, de novo synthesis may not have been established and release was the predominant pattern if denaturation of the mediators can be excluded. The data set of TCA gives the important hint that a total breakdown of the mediators by TCA must be assumed so that concentrations drop below detection limits. A similar point might be taken for SLS with interference to the specific binding sites and preventing a present mediator to bind to its ELISA partner. Here the chemicals tested might introduce at least in the supernatants a new factor of interference allowing only the analysis of relative data.

The production of mediators occurs only by intact or stressed but still vital cells. If denaturation is so severe that most of the cellular body of a cornea (less than 10 % of the total volume) is destroyed, the Kinetic and amplitude of synthesis decreases even if the surviving cells release the maximum amount ever possible. This might reflect elliptical curves of cytokine levels with maximum and minimum medium concentrations due to non stressed cells at low concentration exposures and less surviving cells at high concentration exposures. Therefore the research on linearity might be limited to a certain extent of concentrations affecting only limited areas and leaving the surroundings as intact that stressed cells can react biochemically with synthesis. Here lies on of the a huge differences to living animal experiments where nearby unlimited external supply with leucocytes and macrophages increase the release of mediators.

It is important to keep in mind that the combination of signs of damage like opacification, endothelial necrosis and corneal erosion might be more important than mediator release.

Discussion of the overall project result

We started the project with the task to define a Draize test replacement by a dynamic model close to the animal eye being useful for evaluation of chemicals in simulating a living animal eye. We chose an ex vivo rabbit cornea clamped in a chamber, where the endothelial side was perfused with culture medium and the epithelial side was kept in normal atmosphere. Most important was the ability of recovery within the system and to define differences between various substances being delivered to the model. In the first approach we used controls, mechanical abrasion and four different chemicals (2 molar NaOH, Hydrogenperoxide 3 %, Tomadol 45-7 3 % as non ionic surfactant and Tomadol E 14-5 10 % as cationic surfactant) to demonstrate the reactivity of the system towards different chemicals and the reproducibility within controls and mechanical erosion healing.

We found different patterns of reaction on chemicals in terms of epithelial healing with significant differences between controls and all exposed groups, recovery of an epithelial wound in mechanical abrasion, the incomplete healing with typical pattern of moving epithelial wounds after NaOH and H_2O_2 exposure. These patterns of epithelial regeneration are well known from clinical observation of burnt patients' eyes. Even the time frame of epithelial closure coincides with the healing in human patients. There are typical responses on the corrosive delivery including opacity of the cornea, epithelial break ups, moving erosions and other patterns that are known from eye burn patients and seen in previous work on rabbit eyes. All together, these experiments show that the EVEIT system meets the essential requirements that are imposed on an alternative to the Draize Test, namely independence from living animals, ability to show corneal wound healing after mechanical irritation, the objectivity of the biochemical analyses, different typical patterns of corneal reactivity after burn and similarities to human corneal reactions after comparable irritations. Weak points are the subjectivity of the morphological description, the problems to achieve exactly the same environmental conditions as pH, lactate und glucose levels before eye irritation and the time-consuming setup.

The second step of investigation approached with a smaller amount of ex vivo corneas the question of dose responses on chemicals used in the past as references like Trichloro-acetic acid, Sodiumlaureylsulfate, Sodiumhydroxide and Hydrogenperoxide. Citations are available on these substances with description of dose response in animal experiments of the Draize type. Therefore the two series of each 16 corneas from the first set of experiments were introduced into the dose response studies of the second set.

This approach uncovers the problems of errors due to biological variation of culturing systems with ex vivo organs. Mediator levels as well as the epithelial damage may change. Special properties of tissues like early decontamination of hydrogenperoxide by the endogenous catalase within the corneal epithelium might be responsible for failure of mediator dose responses in low concentrations. Also, IL-8, IL-1 alpha and beta, MIP and VEGF show little responses on low concentrations of irritant chemicals. Nevertheless there is considerable evidence in morphological analysis of epithelial damage and more in endothelial survival over the experimental time, that dose and the specific biological response are detectable within this system. Whether reliable statements on dose response can be made is not to be answered at this very moment, even if the mediator analysis and the systematic research on correlations uncover some linear dose responses, due to the small amount of corneas that were used for these testings and due to the high concentrations of the chemicals that apparently exceeded the maximum efficiency. Nevertheless the results presented show the value of the system and stimulate to develop this system for predictive testing of eye irritation. Further experiments with a bigger number of corneas and different chemical concentrations should be done to verify the option of dose response testings.

Summary

With the objective of replacing the need of living animals to test chemicals respectively their reactions on the eye we created the EVEIT System. Therefore we designed a perfusion chamber in which rabbit corneas of animals that were killed for the food industry were clamped. Their nutrition was guaranteed by a special nutrition medium and the whole setup was stored in a breeder which ensured a constant temperature of 31°C. We did testings in mechanical and chemical ways on these cultivated corneas besides untreated control groups. The corneas were evaluated by morphological (endothelial and epithelial examinations) and biochemical (pH, glucose-lactate-metabolism, cytokines, growth factors in the medium) criteria. We could show that in principle a total healing of the cornea and a recovery of the glucose-lactate-metabolism is possible after trauma. Different chemicals presented varying patterns of trauma and wound healing. The testings with different concentrations of the chemicals give at least a hint of dose response. But further testings with bigger groups of treated corneas and other concentrations need to be done to give better statements.

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Erklärung § 5 Abs. 1 zur Datenaufbewahrung

Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten bei meinem Betreuer, Prof. Dr. Schrage, ACTO hinterlegt sind.

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