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Use of amphoteric rinsing solution for treatment of ocular tissues exposed to nitrogen mustard

Yakov Goldich,[1] Yaniv Barkana,[1] David Zadok,[1] Isaac Avni,[1] Eduard Berenshtein,[2] Mordechai Rosner[3] and Mordechai Chevion[2]

[1]Department of Ophthalmology, Assaf Harofeh Medical Center, Tel Aviv University, Tel Aviv, Israel
[2]Department of Cellular Biochemistry and Human Genetics, Hebrew University-Hadassah Schools of Medicine and Dental Medicine, Jerusalem, Israel
[3]Goldschleger Eye Institute, Sheba Medical Center, Tel Aviv University, Tel Aviv, Israel

ABSTRACT.

Purpose: **Ocular exposure to mustard agents may cause severe and prolonged injury to the anterior segment tissues. Effective decontamination of the external eye surface after exposure is of paramount importance. The purpose of the present study was to assess the effectiveness of Diphoterine rinsing solution (DRS) in reducing ocular damage after exposure to nitrogen mustard (NM) and to compare it with normal saline solution.**

Methods: **One eye of 16 New Zealand albino rabbits was exposed to 2% NM. Immediate thorough irrigation was performed with either 500 ml of DRS (treated group) or with 500 ml of normal saline (control group). The magnitude of ocular injury and response to treatment were assessed by examiners masked to the treatment assignment during 22 days following the exposure.**

Results: **Immediate ocular irrigation with DRS was more effective compared with saline in reducing corneal, iris and anterior chamber injury. In the DRS-treated group, the corneal opacity and corneal neovascularization were less severe, and development of iris atrophy was delayed. Intraocular pressure (mmHg) was better maintained when compared to the control group (day 7 24.3 versus 14.8, $p = 0.003$; day 12 28 versus 15, $p = 0.003$; day 22 33.5 versus 21.8, $p = 0.014$, respectively). Systemic oxidative stress associated with exposure to NM was significantly higher in the saline-treated group than in DRS-treated group ($p < 0.011$).**

Conclusions: **The findings of this study indicate the effectiveness of DRS in reducing of NM-induced ocular injuries. Its use should be considered as an immediate treatment modality following exposure to mustard agents to reduce potential ocular injury.**

Key words: amphoteric rinsing solution – cornea – injury – nitrogen mustard

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Introduction

Chemical warfare has been used in the past and is currently considered a potential weapon by military and civilian defence agencies. Among chemical weapons, mustard agents are among the most powerful yet easy to prepare and use. Their first use was in Belgium in World War I, when one-third of the 1 200 000 who were exposed suffered from prolonged gastrointestinal, dermal, respiratory and ocular injuries. More recently, about three decades ago, mustard agents were widely used against Kurdish civilians and Iranian soldiers during the Iran–Iraq conflict. Chronic effects of exposure on skin, respiratory and ocular systems in Iranian survivors of Iraqi chemical warfare have been recently reported (Rowell et al. 2009). Ocular injuries have been reported to be among the major long-term incapacitating injuries caused by mustard gas, affecting up to 90% of those exposed mainly with chronic and delayed keratopathy (Javadi et al. 2005).

Degree of ocular inflammation following exposure to mustard agent depends on the duration and dose of exposure. Mustard agents act as alkylating agent that induce structural

changes, destruction of nucleic acids and proteins, impairing the cell's normal homoeostasis and eventually causing its death. The toxic chemical reacts rapidly with ocular tissues, and after a latent period of a few hours the patient starts suffering from severe eye pain, photophobia and excessive lacrimation. Initial physical findings include blepharospasm, periorbital oedema, conjunctival injection and inflammatory reaction in the anterior chamber. The intraocular pressure may rise and remain elevated for a few days. After several hours, the corneal epithelium begins to vesicate and slough. In severely injured eyes, there are pupillary constriction, iris vasodilatation, haemorrhages and necrosis with the development of chemical anterior uveitis leading to the formation of posterior synechiae and lens opacification. Late sequelae include corneal scarring and neovascularization as well as neovascular glaucoma (Solberg et al. 1997; Safarinejad et al. 2001).

Currently, there is no known antidote to mustard gas, so washing of the external eye surface after exposure is the mainstay of therapy and of paramount importance (Murray & Volans 1991). Tap water or normal saline historically was used to rinse exposed eyes. However, such solutions provide only passive decontamination by diluting and removing the chemical off the cornea and conjunctiva. A potentially more effective approach would be to combine such flushing activity with active chemical decontamination of the involved chemical agent using amphoteric rinsing solution. Diphoterine rinsing solution (DRS) is an eye and skin decontamination solution that has recently become available for use in emergency rooms in cases of chemical injuries (Hall et al. 2002). Its ability to decontaminate alkylating agents like mustard gas has been shown *in vitro* (Gerasimo et al. 2000).

In an attempt to improve therapeutic options for mustard-induced ocular injury, we sought in the present study to examine the effectiveness of DRS in reducing ocular damage after exposure to nitrogen mustard (NM) and to assess whether it would yield better eye protection compared with saline solution.

Methods

Animal model of ocular NM injury

Sixteen New Zealand albino rabbits weighing 2.5–3.5 kg were used. All animal experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were anesthetized with ketamine HCl (50 mg/kg) injected intramuscularly in combination with the relaxing agent xylazine (5.0 mg/kg). Local anaesthetic drops of oxybuprocaine hydrochloride 0.4% drops (Localin; Fisher Pharmaceutical Labs, Tel-Aviv, Israel) were administered. Ocular mustard injury was induced as previously described by our group (Banin et al. 2003). Briefly, NM (mechlorethamine; Sigma-Aldrich, St Louis, MO, USA), at a concentration of 2% wt/vol, was applied to the cornea of one eye (right) of each animal (the experimental eye) for 5 min within a trephine (Barron radial vacuum trephine; Katena products, Denville, NJ, USA). The vacuum trephine was used to limit the area of application to a circle 6 mm in diameter in the centre of the cornea. Immediately after application, NM was quickly absorbed from within the trephine, with small sponges (Weck-Cel; Medtronicsolan, Jacksonville, FL, USA). The trephine was then removed, and the animal was randomly designated to either treatment or control group (eight each). Accordingly, the injured eye was immediately and thoroughly maskedly irrigated with either 500 ml of DRS or ml of normal saline.

Antibiotic ointment (chloramphenicol 5%) was applied every night to all eyes throughout the follow-up period as detailed below.

At the end of experiment, all rabbits were humanely euthanized using an intravenous overdose of pentobarbital.

Follow-up parameters

The magnitude of ocular injury and response to treatment were assessed by examiners masked to the treatment groups. Repeated slit lamp examinations with scoring of anterior segment injury, measurements of intraocular pressure (IOP), colour photographs of the anterior segment and blood testing of antioxidant status were performed according to the following protocol.

Slit lamp examinations

These were performed before and at 24 h after injury and then repeated after 3, 7, 12, 18 and 22 days. During each examination, the following parameters were recorded:

(1) Area of corneal epithelial loss (corneal erosion): The average horizontal and vertical linear dimensions of the epithelial defect as stained by locally applied fluorescein were measured using the adjustable slit lamp beam and the area computed in mm^2.
(2) Degree of corneal opacity: grade 0-clear cornea with details of iris observed clearly; grade 1-mild blurring of iris details; grade 2-moderate opacity with blurred iris crypts; grade 3-severe corneal opacity, no iris details visible.
(3) Degree of iris pigmentation: grade 0-no pigmentation; grade 1-mild, grade 2-moderate and grade 3-severe iris pigmentation.
(4) Degree of iris atrophy: grade 0-no atrophy; grade 1-sectoral atrophy; grade 2-total atrophy.
(5) Degree of corneal neovascularization (CNV): grade 0-no neovascularization; grade 1-mild, up to three clock hours of corneal neovascularization; grade 2-moderate, up to 6 hr and grade 3-severe, more than six clock hours of corneal neovascularization.
(6) Degree of iris neovascularization: grade 0-no neovascularization; grade 1-mild neovascularization; grade 2-moderate; grade 3-severe neovascularization.
(7) Additional observations recorded included extent of eyelid and conjunctival swelling and injection; cataractous changes; anterior chamber reaction; and, when present, hyphema and corneal perforation.

Intraocular pressure

Intraocular pressure measurements were performed in treated eyes with a hand-held automated tonometer (Tono-Pen AVIA applanation tonometer; Reichert, Buffalo, NY, USA) after installation of local anaesthetic

drops. Baseline IOP was measured before NM exposure and re-measured on days 1, 3, 7, 12, 18 and 22 days after injury.

Colour photographs

Colour photographs of the anterior segment were taken at 1–22 days after injury using a hand-held slit lamp-mounted photocamera (Pentax Optio S60, Golden, CO, USA).

Systemic antioxidant status

Systemic oxidative stress was assessed using oxidative stress by ascorbic acid (OSAA) method (Chevion et al. 1999). Ascorbic acid (AA), a naturally occurring free radical scavenger, is normally present in the blood. Before the injury and at days 1, 7 and 22 of the experiment blood samples were obtained, and systemic oxidative stress was assessed by measurement of AA dissipation. AA, the reduced form of ascorbate and the fraction of the oxidized ascorbate (dehydro-ascorbate, DHAA) were measured (Motchnik et al. 1994), and the ratio expressed in percentage between them was calculated and denoted as OSAA (%): OSAA = (DHAA × 100)/(AA + DHAA) (Chevion et al.1999).

Histology of ocular structures

On the last day of the experiment, eyes of two animals from each group were obtained for histologic examination. Eyes were enucleated and fixed in 4% paraformaldehyde. After specimens were embedded in paraffin, 4-μm sections were cut and stained with haematoxylin and eosin for histologic evaluation of injury.

Statistical method

Means and standard deviations were calculated for all parameters in the two groups for each measurement.

A one-way (group) analysis of variance (ANOVA) with repeated measures (over time) was performed to assess the difference in time trend between the two groups in the various parameters. Pairwise comparisons between each time point and Baseline comparison and comparison between groups for each such difference were performed using contrast analysis.

For IOP, separate analyses of variance were performed for each group to assess differences between each time point and baseline measurement.

Adjustment for significance level was performed using Sidak method.

All statistical analyses were performed using SAS (SAS Institute Inc., USA) for Windows 9.2.

Results

The experimental exposure of rabbit eye to NM caused severe and prolonged injury to the ocular structures and had a systemic oxidative effect.

Corneal erosion

One day after injury with NM, the corneal epithelial defects of comparable size were noted in both groups (15.8 ± 8.0 mm^2 versus 16.6 ± 8.6 mm^2, respectively, $p = 0.86$). Corneal epithelial defects resolved completely by day 3 in both groups.

Corneal opacity

Figure 1A describes the mean corneal opacity score in both groups during the experiment. Starting the first day after the mustard injury, the average score of corneal opacity in control saline-treated group was significantly higher than in DRS-treated group (Day1: $p < 0.001$, Day 3: $p = 0.012$, Day 12: $p = 0.024$, Day 18: $p = 0.007$, Day 22: $p = 0.001$). The only time point differences between groups were not statistically significant is on Day 7 when we observed the slight increase in opacity score in DRS-treated group coincidental with slight decrease in this score in the control group ($p = 0.1$).

Corneal neovascularization

Neovascularization of cornea was developed to various degrees in both experimental groups after the mustard injury (Fig. 1B). The first neovascular vessels were observed on Day 7 in both groups. Starting day 18, the control group showed a statistically significant increase in corneal neovascularization in comparison with the DRS treatment group (Fig. 2) during the observation period (day 18 $p = 0.029$; Day 22 $p = 0.034$).

Iris changes

Exposure to mustard agent caused increased iris pigmentation, iris atro-

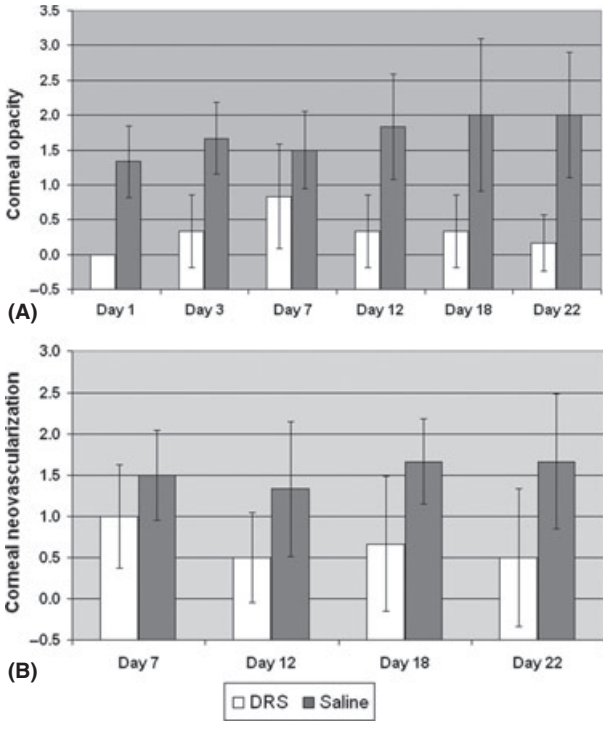

Fig. 1. (A) Corneal opacity score (mean ± SD) after exposure to 2% nitrogen mustard (NM) in both study groups. (B) Corneal neovascularization score (mean ± SD) after exposure to 2% NM in both study groups.

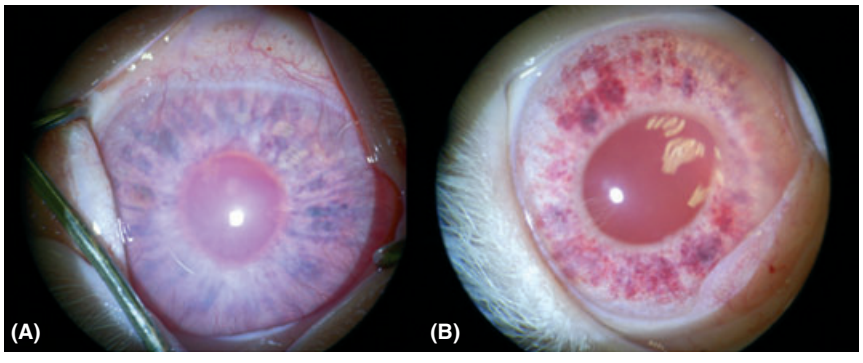

Fig. 2. Slit lamp photographs of the anterior segment at day 22. Note conjunctival haemorrhages, corneal opacity and corneal neovascularization more pronounced in saline-treated group (A) than in the diphoterine rinsing solution-treated group (B).

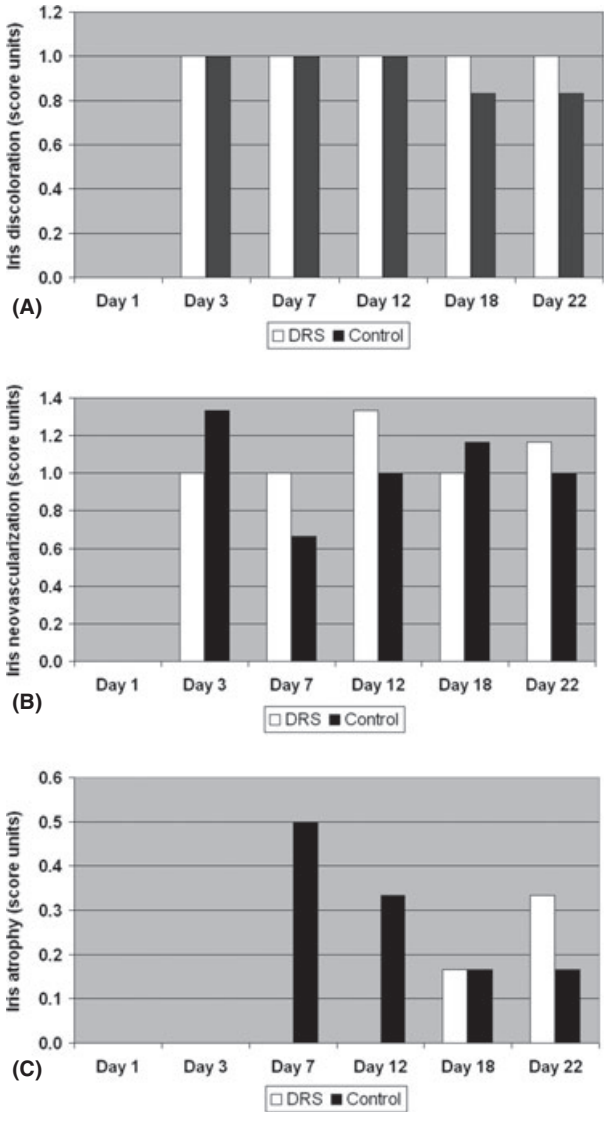

Fig. 3. Iris pigmentation, iris neovascularization and iris atrophy scores after exposure to 2% nitrogen mustard in both study groups.

phy and iris neovascularization in both experimental groups (Fig. 3).

Increased iris pigmentation was first noted on Day 7 time point in both groups and remained throughout the experiment period without statistically significant difference between the groups. Iris neovascularization was first noted on Day 3 in both groups and remained throughout the experiment period without statistically significant difference between the groups. Iris atrophy was first noted on Day 7 in saline-treated group and continued to exist throughout the examination period. In the DRS-treated group, iris atrophy appeared much later, on Day 18. When both groups showed such atrophy, the calculated iris atrophy score did not show a statistically significant difference between the groups.

Intraocular pressure

Figure 4A describes the average IOP in both groups before and following ocular exposure to NM. The preexposure IOP, measured before the randomization, was statistically similar in both groups ($p = 0.936$). Following the NM exposure, an increase in IOP occurred on Day 1 in both groups and remained elevated throughout the experiment period. Contrast analysis for the saline-treated group showed that postexposure IOP values in the saline-treated group were statistically significantly higher than the preexposure IOP at all time points. The same analysis for the DRS-treated group revealed significant differences between preexposure value and postexposure values on Day 1, Day 18 and Day 22. Mean IOP was statistically significantly higher in the DRS group compared with the saline group on day 7 (24.3 mmHg versus 14.8 mmHg, respectively, $p = 0.003$), day 12 (28 mmHg versus 15 mmHg, $p = 0.003$) and day 22 (33.5 mmHg versus 21.8 mmHg, $p = 0.014$).

Systemic antioxidant status

Results are shown in Fig. 4B. On Day 1, a statistically significant increase in OSAA was measured in both DRS and saline-treated groups ($p = 0.011$ and $p < 0.001$, respectively). In the saline-treated group, OSAA showed a tendency to decrease following Day 1, but still remained significantly higher, as compared to the preexposure values (day 7 and day 22, $p < 0.0001$). In the DRS-treated group, after the observed peak at Day 1, the OSAA gradually declined but remained significantly higher then the preexposure values at day 7 ($p = 0.011$) and day 22 ($p = 0.042$). Increased OSAA, that

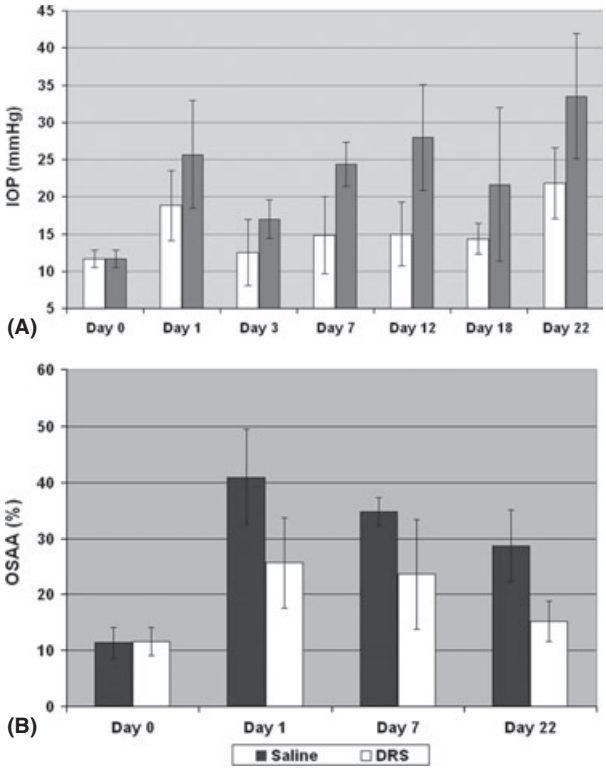

Fig. 4. (A) Intraocular pressure (mean ± SD) before and after exposure to 2% nitrogen mustard (NM) in both study groups. (B) Systemic oxidative stress as assessed by ascorbic acid (OSAA) presented as mean ± SD of results obtained from blood samples from both study groups before and after exposure to 2% NM.

reflects the severe systemic oxidative stress associated with exposure to NM, was significantly higher in the saline-treated group compared with the DRS-treated group throughout the experiment (day 1, $p = 0.007$; day 7, $p = 0.011$; day 22, $p = 0.001$).

Ocular histology

On day 22 after exposure to NM the injured eyes of two animals from each study group were obtained for histological assessment (Fig. 5).

Corneal oedema and neovascularization were more pronounced in the saline-treated eyes (D) compared with the DRS-treated eyes (A). Iris intrastromal haemorrhages were similar in both groups (B and E), but advanced neovascularization was noted predominantly in the saline-treated eyes (E). Cataractous lens changes with formation of morgagnian globules were more evident in the saline-treated eyes (F) than the DRS-treated eyes (C).

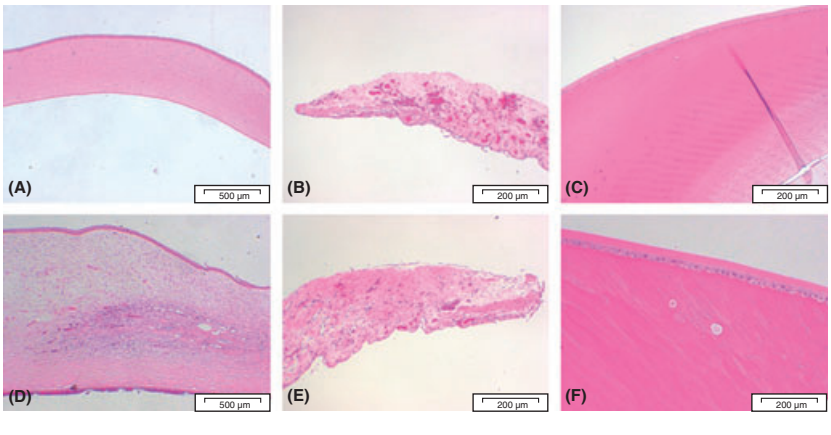

Fig. 5. Histology preparations stained with hematoxylin and eosin. Cornea (A, D), iris (B, E), and crystalline lens (C, F) injury at Day 22 after exposure to NM. (A–C) DRS treated group and (D–F) saline treated group.

Discussion

In the present study we demonstrated that immediate ocular irrigation with DRS following corneal exposure to NM was more effective than immediate irrigation with saline in reducing corneal injury, IOP elevation and systemic oxidative stress.

Ocular exposure to mustard agents may cause severe acute and prolonged injury to the anterior segment tissues (Solberg et al. 1997; Safarinejad et al. 2001; Banin et al. 2003; Kadar et al. 2009). Lack of known specific antidote to mustard gas emphasize the importance of effective decontamination of external eye surface following the noxious exposure to mustard agent (Murray & Volans 1991). Hypotonic or iso-tonic solutions like tap water or normal saline are most available for ocular irrigation after the exposure. However, such solutions provide only a passive decontamination by washing the chemical off the cornea and conjunctiva. A better approach would be to combine such flushing activity with active chemical decontamination of the involved chemical agent using hypertonic amphoteric rinsing solution. In our study we used Diphoterine rising solution for immediate ocular irrigation following eye exposure to NM. DRS is a polyvalent, hypertonic (osmolarity 820), neutral (pH = 7.4), amphoteric, water-soluble compound, which binds acids, bases, oxidizing agents, and solvents (Hall et al. 2002). Constituents of DRS include Diphoterine molecule, sodium chloride, glycine, preservatives and distilled water; and accordingly to the manufacturer its activity involves three modes of action. As a liquid irrigation solution it mechanically removes noxious surface contaminants. As a chelating molecule it binds various chemicals including acids, alkali, irritants, solvents, radionuclides, organophosphates and alkylating agents such as mustard gas. As a hypertonic solution, it impedes chemical tissue penetration and removes some amount of cornea-absorbed toxicants that have not already bound to the tissue (Hall et al. 2002). DRS use was previously reported as a skin and ocular treatment following chemical

injuries. Merle et al. (2005) compared effectiveness of DRS and saline for emergency use following alkali ocular burn in Martinique population. They noted that reepithelialization time was shorter in group treated with the DRS. Schrage et al. (2002) used *in vivo* rabbit model to assess DRS effectiveness in treating ocular alkali injury. Measuring intra anterior chamber pH they showed that DRS was more effective than saline in terms of buffering capacity following NaOH injury. Gérasimo et al. *in vitro* studied DRS ability to decontaminate skin fragments exposed to mustard agent. They reported that DRS was more effective than water with soap or physiologic saline in decontaminating human skin fragments following the *in vitro* exposure to sulphur mustard (Gerasimo et al. 2000).

The results of our study shows the effectiveness of immediate ocular irrigation with DRS following corneal exposure to NM in reducing corneal, iris and anterior chamber injury and systemic oxidative stress. In the cornea we observed significantly reduced opacity and neovascularization in DRS-treated group. Elevated IOP as a possible indicator of aqueous humour outflow compromise was significantly higher in saline-treated group. Initial iris atrophy was delayed from day 7 in the saline-treated group until Day 18 in the DRS-treated group.

Systemic oxidative stress after ocular exposure to mustard agents was previously described (Kadar et al. 2001; Banin et al. 2003). In both our experimental groups we observed induced systemic stress with similar time pattern, but in the DRS-treated group the increase in OSAA was significantly less and return to baseline levels was faster than in the saline-treated group. These results may imply that DRS efficiently reduced the total quantity of NM that penetrated the corneal stroma and induced acute and prolonged injury. Although irrigation with DRS did not completely prevent ocular damage, the treatment results were substantially better than in the group treated with saline solution.

The relatively short follow-up time is an obvious weakness of our study. As mustard agents are known to cause delayed injury effects even after months and years, some long-term studies may be needed in this regard.

In summary, DRS was found to be more effective than saline in the treatment of mustard ocular injuries.

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Correspondence:
Yakov Goldich, MD
Department of Ophthalmology
Assaf Harofeh Medical Center
Zerifin 70300
Israel
Tel: 972 8 977 9358
Fax: 972 8 977 9357
Email: doctor.goldich@gmail.com