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In vivo and *in vitro* irritation testing of low concentrations of hydrofluoric acid

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Abstract

Objective. Acidic fluorides are proposed for the treatment of dental erosion. The aim of this study was to examine the irritation properties of dilute hydrofluoric acid (HF) solutions for potential use in the oral cavity. ***Material and methods.*** Hen's egg test–chorioallantoic membrane (HET-CAM): The CAM was accessed by careful dissection through the egg shell ($n=36$, 6 eggs/test solution) and exposed to 300 µl of the HF solutions (0.05%, 0.10%, 0.20%, and 1.0%) under macroscope examination over the course of 5 min. Mean time-to-coagulation and average irritation score were recorded based on appearance of hemorrhage, coagulation, and lysis of the blood vessels in the membrane. Mouse skin test: 60 male mice were randomly divided into 10 groups of 6 animals each (control, 0.05%, 0.10%, 0.20%, and 1.0% HF), shaved on the back, exposed to test solution, and euthanized after 2 h or 24 h. Skin samples were evaluated by light microscopy, scoring epithelial leukocyte infiltration, vascular congestion, and edema. ***Results.*** HET-CAM: 0.05% HF was slightly irritant, 0.1% HF moderately irritant, 0.2% and 1% HF strongly irritant. 0.1–1% HF solutions were severely irritating on the eye. Mouse skin test: HF concentration was significantly correlated with tissue response, and 24-h exposure to 1% HF solution showed focal erosion of the epithelium and marked localized subepithelial leukocyte infiltration. ***Conclusion.*** The results of the studies suggest that accidental exposure of soft tissues to solutions containing more than 0.2% HF may be harmful.

Key Words: *Animal study, corrosive, HET-CAM, hydrogen fluoride, HF*

Introduction

Tooth wear, a multifactorial phenomenon, is a common problem in many countries today. It is well known that exposure to intrinsic and extrinsic acids is a major contributing factor and may lead to erosive tooth wear over time [1,2]. Methods for treatment and prevention of dental erosion have been studied extensively over the past couple of decades. Sodium fluoride, used for remineralization of dental caries lesions, is ineffective at low pH levels (pH$<$4.5) [3], implying that standard fluoride prophylaxis has little or no effect on tooth erosion. However, more recent studies have shown that acidic fluoride solutions reduce the enamel dissolution resulting from acidic challenges [4–8]. Hydrofluoric acid (HF) is the simplest form of acidic fluoride and other acidic fluoride solutions contain HF [9].

The concept of focusing on HF as a means, or a mechanism, for reducing erosive tooth wear is controversial. HF is a well-known toxicant and corrosive agent [10–12], and even at low concentrations HF solutions cause dermal and ocular damage following exposure [11]. Recent studies using an experimental *in vivo* dental erosion model have indicated that dilute HF solutions ($\leq$0.2%) can reduce enamel dissolution when teeth are exposed to a citric acid challenge [4]. However, prior to any widespread experimental use of HF in humans, the relevant concentrations should be evaluated in suitable biological test systems to examine for possible hazards. When HF is applied to tooth surfaces in a clinical setting there is a real possibility of accidental spillage of the solution on to the oral mucosa, and testing for irritation of the oral mucosa is relevant. This situation also applies in the case of

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other potential irritants currently used in clinical dentistry.

The hen's egg chorioallantoic membrane (HET-CAM) test [13,14] has been used successfully to evaluate the irritation potential of facial prosthesis adhesives on the skin and dental materials on the oral mucosa [15,16]. In addition, a mouse skin model was developed for our purposes to further evaluate the possible irritation effect of dilute HF solutions using a true *in vivo* test system. The aim of this study was therefore to examine the irritation effects of four dilute HF solutions using the HET-CAM test and a mouse skin model.

Material and methods

Test solutions

The following four concentrations of HF were tested in both studies, i.e. 0.05%, 0.1%, 0.2%, and 1%, produced by diluting 40% HF (Rectapur, Prolabo, Paris, France) in deionized water. A positive control (0.10% NaOH; Sigma-Aldrich, Steinheim, Germany) and negative control (0.9% NaCl; VWR International, Fontenay sous Bois, France) made using deionized water were included in the HET-CAM test; deionized water was the negative control in the mouse skin test.

HET-CAM test

A modified version of the HET-CAM test, as described by Kalweit et al. [13], was carried out on fresh, fertilized hens' eggs that had been incubated for 9 d. Any non-viable embryos were discarded before testing. A total of 36 eggs were used, i.e. three eggs were used for each test solution and the controls, and the entire test set-up was repeated once on a second day. The chorioallantoic membrane was first accessed by careful dissection. The air cell was marked with a rotating dental instrument, the shell was removed, and the air cell accessed. The inner membrane was then moistened with saline solution and carefully removed to expose the chorioallantoic membrane. 300 µl of the test fluid or control was dripped on the membrane under examination using a macroscope (Wild M400; Wild Heerbugg, Switzerland). The type of reaction in the membrane (hemorrhage, lysis, and coagulation) and the time to the appearance of any of the reactions up to a maximum of 300 s were recorded. The average irritation score (IS) was calculated using the following formula:

$$\mathrm{IS} = \frac{301 - \mathrm{H}}{300} \times 5 + \frac{301 - \mathrm{L}}{300} \times 7 + \frac{301 - \mathrm{C}}{300} \times 9$$

H = time (s) of first appearance of hemorrhage, L = time (s) of first appearance of lysis, C = time (s) of first appearance of coagulation. The membrane was observed at the following intervals: 30, 60, 120, 240, and 300 s after exposure. A score of 301 was assigned when no reaction was seen after 300 s. The IS score for each solution was reported as the mean for all six eggs, and the solutions were classified according to Kalweit et al. [13]. IS 0–0.9 = non-irritant, IS 1–4.9 = slight irritant, IS 5–8.9 = moderate irritant, and IS 9–21 = strong irritant. Mean time to coagulation (MTC_{100}) was also calculated. Solutions having MTC_{100} values <100 were classified as severely eye irritating, corresponding to risk sentence #41 (R41) in the labeling of hazardous chemicals [17]. After testing, the eggs were placed in a freezer ($<-20^{\circ}C$) in order to euthanize the embryos.

Mouse skin test

A total of 60 male mice (C57BL/6JbomTac) were randomized into two groups: the 2-h group and the 24-h group, where 6 animals were used for each of the 4 concentrations of HF. The mice in the 2-h group were 9 weeks old with an average weight of 27.6 g, while those in the 24-h group were 8 weeks old with an average weight of 25.6 g. The animals were anesthetized with halothane gas and a shaved patch of skin was prepared on the back of the mice with an electric razor. 300 µl of the test solution was pipetted into a plastic dappen cup and applied with a brush (Duncan TB 731 No. 7 round; Duncan Enterprises, Calif., USA) to the shaved area for approximately 15 s. The mice were kept separated from each other until the test solutions were fully absorbed and their condition checked at regular intervals until euthanized. To ensure the welfare of the animals, the experiments were to be aborted and the animals euthanized if any signs of major discomfort, distress, or pain were to occur during the observation period. The animals were euthanized after 2 h and 24 h, respectively, using CO_2 gas, and the exposed skin was evaluated by the naked eye, dissected, removed, and pinned to a cork plate in order to prevent the specimen from curling. The skin samples were placed in 12 ml buffered 4% formalin made from 36% formaldehyde (Merck, Briare-le-Canal, France) and stored at room temperature. The buffer was made from sodium dihydrogen phosphate (Merck) and disodium hydrogen phosphate (Prolabo). The test was carried out at the National Institute of Public Health, Oslo, Norway. The mice experiment was approved by the local responsible laboratory animal science specialist under the surveillance of the National Experimental Animal Board in Norway and registered by the board.

The skin samples were removed from the corks after 3 d of fixation and the skin left for another 4 d in 12 ml of buffered 4% formalin before tissue processing starting with a water rinse, followed by a

graded series of ethanol, xylene, and paraffin (Citadel 2000; Thermo Shandon, Mass., USA), and, finally, sectioning into 5 μm thick serial sections perpendicular to the surface, stained with hematoxylin and eosin and evaluated by light microscopy (Olympus model BX 51TF; Olympus Corporation, Tokyo, Japan) at 400 times magnification. Epithelial leukocyte infiltration, vascular congestion, and edema were scored according to a modification of the ISO standard 10993-10:2002 grading system [18] for microscopic examination of oral, penile, rectal, and vaginal tissue reactions. The following parameters were recorded:

- Epithelium: normal intact, cell degeneration or flattening, metaplasia, focal erosion, generalized erosion.
- Leukocyte infiltration: absent, minimal (<25 cells), mild (26–50), moderate (51–100), and marked (>100) in a 400× field using the region of the section with the highest leukocyte infiltration.
- Vascular congestion: absent, minimal, mild, moderate, marked with disruption of vessels.
- Edema: absent, minimal, mild, moderate, and marked.

Exposure and evaluation of the sections were both performed blind with regard to test solutions and groups.

Statistical analysis

The results for the HET-CAM test (irritation score and mtc_{100}) and for the mouse skin test (leukocyte infiltration and epithelium reaction) were analyzed using SPSS version 15.0 software (SPSS, Chicago, Ill., USA) with Spearman's rank-order correlation test to identify whether there was any correlation between the concentration of test solution and tissue response.

Results

HET-CAM test

The results are given in Table I and shown in Figure 1. The 0.05% HF solution gave a response that was classified as a slight irritant, 0.1% HF as a moderate irritant, while both 0.2% and 1% HF were classified as strong irritants. Mean time to coagulation (mtc_{100}) for 0.1%, 0.2%, and 1% HF was <100 s, i.e. classified as R41 severely eye irritating. Spearman's rank-order correlation test showed a statistically significant correlation between the concentration of HF and the response of the chorioallantoic membrane (irritation score and mtc_{100}) ($r_s = 0.878$, $p < 0.0005$).

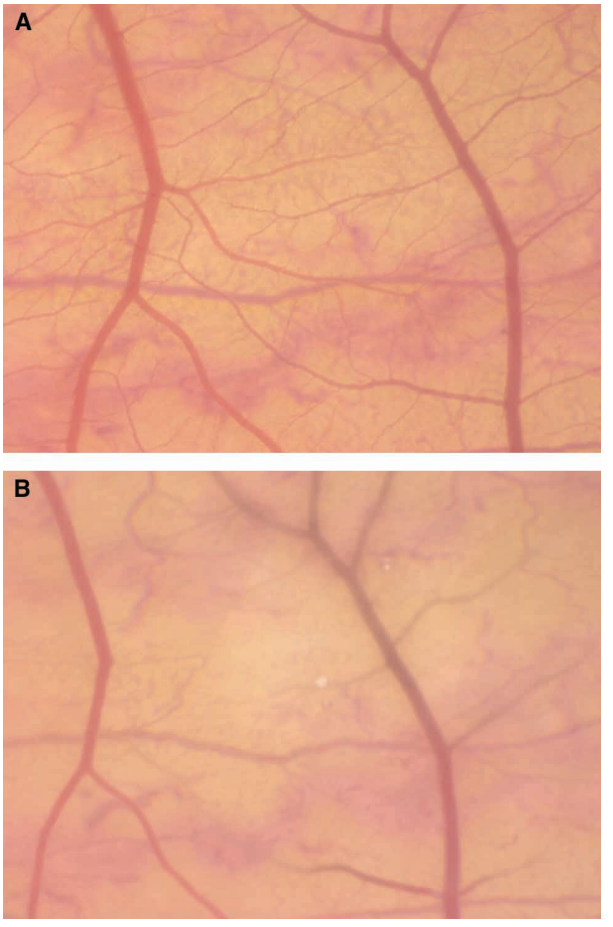

Figure 1. Photomacroscopic images of the chorioallantoic membrane (CAM) of a hen's egg showing the blood vessels prior to exposure (A) and the same area 240 s after (B) exposure to 0.2% hydrofluoric acid. The lysis (disappearance) and coagulation of blood vessels in (B) compared to (A) are clearly visible.

Mouse skin test

The results are presented in Table II and Figure 2. Clinically, the animals in the 24 h 1% HF solution

Table I. Irritation score (IS), classification and mean time to coagulation (mtc_{100}) for the Hen's Egg Test-chorioallantoic membrane (HET-CAM) test.

Solution	Main insult	Other insult	mtc_{100}	IS	Description
0.9% NaCl (negative control)	No reaction			0.00	Non-irritant
0.05% HF	Coagulation	Hemorrhage/lysis	190	3.26	Slightly irritant
0.1% HF	Coagulation	Lysis	75	7.49	Moderately irritant
0.2% HF	Coagulation	Lysis	80	9.92	Strongly irritant
1.0% HF	Coagulation	Hemorrhage/lysis	50	12.22	Strongly irritant
0.1% NaOH (positive control)	Hemorrhage	Lysis/coagulation	50	16.42	Strongly irritant

Table II. Results of the mouse skin test showing the number of mice where leukocyte infiltration and focal erosion were observed.

	Mice with leukocyte infiltration (*n*)					Mice epithelium reaction (*n*)	
Solution	Absent	Minimal	Mild	Moderate	Marked	Absent	Focal erosion
2 h							
H_2O		6				6	
% HF: 0.05	4	2				6	
0.1		5	1			6	
0.2	1	2	2	1		6	
1.0		1	5			6	
24 h							
H_2O	1	4	1			6	
% HF: 0.05		5	1			5	1
0.1	2	4				6	
0.2	1	4	1			6	
1.0			1	1	4	1	5

group showed signs of local inflammation (red and swollen skin). When removing the skin from these mice, bleeding was noted in the underlying tissue in some animals; one animal had skin damage throughout the skin layer. These animals also seemed to be in poorer general health than those in the other groups by the time they were euthanized. This change in health status appeared to occur just prior to termination of the observation period. No vascular congestion or edema was observed in any sample. When present, leukocyte infiltration and focal erosion were localized (Figure 2).

There was a significant correlation between the test solution concentration and the tissue response in regard to leukocyte infiltration for both mice groups at 2 h and at 24 h ($r_s = 0.575$ and 0.485, respectively) ($p < 0.0005$). Focal erosion was not observed after 2 h, whereas after 24 h there was a significant correlation between the test solution concentration and the presence of focal erosion ($r_s = 0.530$, $p < 0.0005$).

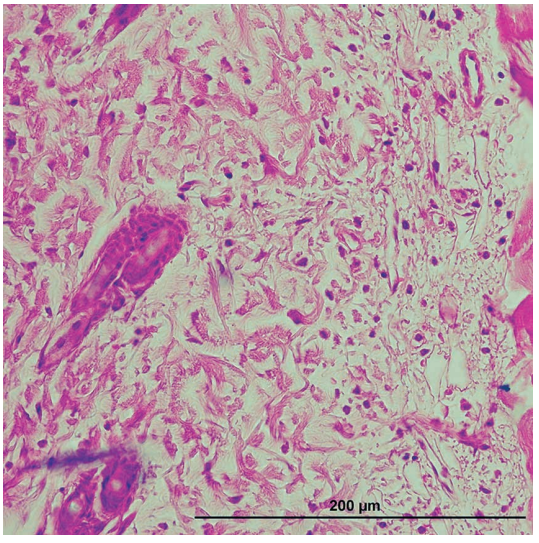

Figure 2. Histological image of leukocyte infiltration in mouse skin 24 h after exposure to 1% hydrofluoric acid using H&E staining and light microscopy.

Discussion

The present study investigated the possible tissue/host response after contact with HF at the same very low concentrations that have been tested in an experimental *in vivo* dental erosion model [4]. Although the results of the HET-CAM test showed that all of the HF concentrations tested induced some degree of injury, a clear dose–response relationship was observed. In the mouse skin test, the results showed a clear dose relationship regarding leukocyte infiltration. A similar dose–response relationship has also been observed for more concentrated HF solutions [11].

Based on their ability to damage the blood vessels, the weakest HF solution had minimal detrimental effects. In contrast, the three strongest HF solutions could be classified as severely eye irritating, according to Spielmann et al. [19]. However, these high scores are similar to what was found by Dahl when testing frequently used dental adhesive agents (primer and bonding material) in the same model [16,19]. The vast majority of the tested substances were scored as moderate to strong irritants, and with a few exceptions most of the tested materials were classified as severely eye irritating [17]. This led Dahl [19] to conclude that dental adhesives have the potential to cause an irritant reaction in oral mucosa, and that care must be taken when applying these agents to tooth surfaces in the oral cavity.

The basis for the present study is that acidic fluorides have been shown to be more effective than neutral fluorides in the prevention and treatment of dental erosion [4,20,21]. The simplest acidic fluoride, hydrogen fluoride, comprises very small molecules and is naturally present in fluoride solutions with low pH levels, either as the non-dissociated species (H-F) or in the ionized form [22]. It has been suggested that hydrogen fluoride is important in reducing the solubility of dental hard tissues, and recent studies using an experimental *in vivo* dental erosion model indicate that application of hydrogen

fluoride solutions to the dental enamel can increase the tooth surface resistance to further acid attack by a typical dietary acid [4]. Theoretically, low-concentrated HF solutions could therefore potentially have clinical applications in cases of dental erosion. However, although commonly used agents such as stannous fluoride or acidic phosphate fluoride (APF) contain HF, this is a corrosive agent and alone would not normally be considered as a suitable treatment agent for use in the oral cavity. Solutions of approximately 10% HF are currently being used for etching ceramic dental restorations as a means of increasing the bond strength between the fitting surfaces of the restoration and the composite resin cements (9.5% HF; Ultradent Products Inc., South Jordan, Ut., USA). There is no doubt that HF at such high strength is highly toxic, with the potential to cause both local and systemic injury [11]. HF solutions as dilute as 0.01% have been reported to cause visible skin lesions after 5 min exposure in an albino rabbit model [23].

The HET-CAM test was developed to replace the Draize eye test for severely eye irritating chemicals during the late 1980s and early 1990s [17]. Over the years, it has also proved suitable for testing dental materials with regard to the potential to cause an irritant reaction in oral mucosa [16,19]. It is a test with high reproducibility, bordering between *in vivo* and *in vitro* testing, and in the present context was considered relevant for our purposes.

Regarding the mice skin model results, leukocyte infiltration was seen in the dermis in all groups, including the control group. It is probable that shaving the back of the mouse alone may have caused some leukocyte infiltration. In the 24 h group, both focal erosion of the epithelium and the most marked leukocyte infiltration were observed in the skin after exposure to the most concentrated HF solution (1.0% HF). The results indicated that this concentration gave an erosive response in the mouse skin and that it may also be harmful for human skin or oral mucosa. It was more difficult to observe any clear difference in tissue response for the other HF concentrations (0.05–0.2%). Although there are several obvious differences between mouse skin and human oral mucosa, these results indicate the potential irritating effect. Intra-oral testing on suitable test animals, such as hamsters, could provide even more relevant results than obtained in this mouse skin model.

The standardized scoring system of the ISO 10993-10:2002 used for the mice skin test was initially developed for oral, penile, rectal, and vaginal tissue, and was therefore modified for our purpose. All skin samples included areas without any tissue response, and the area with the highest score of leukocyte infiltration was recorded in order to have a worst case scenario. There were no cases of general inflammation, the leukocyte infiltration being localized and focal. This observation may be explained by the small amount of solution used and/or the short exposure time. There were also some differences in response among animals within the same group that can be explained by biological variability. Furthermore, some animals may have woken up and moved around before all of the solution was absorbed into the exposure area, thereby affecting the dose.

Hydrogen fluoride in water, HF, differs from other aqueous acidic fluoride solutions such as titanium fluoride and stannous fluoride in that it contains no metal ions, but hydrogen fluoride both in the ionized and non-dissociated form. Stannous fluoride and titanium fluoride contain only the ionized form of HF. Non-dissociated hydrogen fluoride is smaller than the fluoride ion and will most likely cross lipid membranes and penetrate tissues more rapidly. The transport of hydrogen fluoride through biological membranes has been shown to occur mainly by non-ionic diffusion [24], and in the present study presumably resulted in rapid penetration of the blood vessels. The fact that HF solutions have a very low pH ($pK_a = 3.20$) [25] may go some way to explaining the reactions in the chorioallantoic membrane and in mice skin. These qualities and behavior of HF may be useful when attempting to strengthen the dental enamel either as a form of treatment or as a preventive method in clinical cases of dental erosion. While ionic fluoride can be absorbed into the crystal surface of the enamel and possibly replace the hydroxyl groups in the hydroxyapatite lattice, non-dissociated HF molecules may diffuse into the inter-crystalline spaces of enamel, thereby providing a more acid resistant structure [22,26].

It can be concluded from this study that although low concentrations of HF in the range 0.05–1% were observed to have irritation potential, both *in vitro*, on the chorioallantoic membrane of hens' eggs, and *in vivo*, on mouse skin, similar reactions have been described for dental materials currently in use. Within the limits of this study, the results suggest that exposure of soft tissues to solutions containing more than 0.2% HF may be harmful.

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