Corneal Endothelial Toxicity of Air and SF6

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PURPOSE. The authors conducted in vivo assessment of corneal endothelial toxicity of air and SF6 in the feline model. This research was motivated by the increased use of air in anterior segment surgery in human subjects.

METHODS. This was a prospective masked study. The eyes of 16 healthy adult cats were randomly assigned for the injection of 0.7 ml air into the anterior chamber of one eye and SF6 in the contralateral eye. Daily examination included slit lamp photography, pachymetry, and tonometry. Specular microscopy was performed before, 7 days after, and 10 days after injection. The animals were euthanatized, and the corneas were processed for alizarin red trypan blue staining and for light and electron microscopy.

RESULTS. SF6 remained in the anterior chamber significantly longer than air. Both groups showed postinjection inflammation, which on average was maximal at day 2 and more severe with SF6. No difference in IOP was observed between the two groups. Specular microscopy showed significant endothelial cell loss in the SF6 group (mean postinjection cell loss, 132 ± 50 cells/mm²) but not in the group injected with air. Alizarin red staining revealed significant regional differences in cell density only in the SF6 group and more pronounced endothelial cell loss in the superior area.

CONCLUSIONS. These results indicate that both air and SF6 injected into the anterior chamber of the eye can induce intraocular reaction in the feline model and that SF6 is more toxic than air in terms of endothelial cell loss and anterior chamber inflammation. (Invest Ophthalmol Vis Sci. 2011;52:2279-2286) DOI:10.1167/iovs.10-6187

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Injection of air into the anterior chamber of the eye has become standard practice in anterior segment surgery. The air bubble is used to exert direct pressure on an intraocular structure. Because air is less dense than aqueous humor, the bubble remains upward. The patient is asked to maintain the eye position that will optimize contact between the air bubble and the tissue layer to be stabilized. In Descemet stripping automated endothelial keratoplasty (DSAEK), air is used to promote donor tissue adherence to the recipient bed, thus eliminating the need for sutures. The graft is centered over the posterior surface of the cornea, and the patient is asked to lie in a supine position and to look straight at the ceiling for 1 hour after surgery to distribute pressure from the air bubble over the graft surface. Similarly, the standard treatment for graft dislocation after DSAEK consists in air re-injection with head positioning. Air can also be used to tamponade a detached Descemet’s membrane after trauma or complicated cataract surgery. For superior detachment, the patient is asked to keep his head in the upright position. Air has also been used recently to shorten the period of corneal edema when hydrops develops in keratoconus eyes. Sulfur hexafluoride (SF6) has been proposed as an alternative to air because it remains in the anterior chamber for a longer period. Although now routinely used in several anterior segment surgeries, the safety of air and SF6 for the corneal endothelium still must be demonstrated. The purpose of this study was to compare the in vivo corneal endothelial toxicity of air and SF6 in the feline model.

METHODS

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the tenets of the Declaration of Helsinki. This research protocol was approved by the Maisononneuve-Rosemont Hospital Committee for Animal Protection (Montreal, QC, Canada).

Population

Sixteen healthy adult cats (mean weight, 5.06 ± 0.44 kg; range, 2.2–8.6 kg) were obtained from a certified supplier. One eye of each animal received an intracameral injection of 20% (nonexpansive mix)7 sulfur hexafluoride (SF6) (Labtician Ophthalmics Inc., Oakville, ON, Canada), and the other eye received a similar volume of filtered air. The choice of the eye was randomized, and all observers were masked for all steps (injection, daily examination, and endothelial cell morphometric analysis).

Surgical Protocol and Medication

Surgery was performed under general anesthesia. Each animal was premedicated with an intramuscular injection of premixed acepromazine (0.05 mg/kg; Sanofi Aventis, Laval, QC, Canada), glycopyrrolate (0.01 mg/kg; Axcan Pharma, Mont Saint Hilaire, QC, Canada), and buprenorphine (0.01 mg/kg; Schering-Plough, Kirkland, QC, Canada) and was intubated. Anesthesia was induced and
maintained by inhalation of isoflurane 2% (Baxter, Mississauga, ON, Canada). Atracurium (0.25 mg/kg, followed by 0.1 mg/kg every 20 to 30 minutes as needed; Sandoz, Boucherville, QC, Canada) was used to induce paralysis of the extracocular muscle. Full pupil dilatation was obtained with topical administration of tropicamide 1% (Alcon, Mississauga, ON, Canada), phenylephrine 2.5% (Alcon), and cyclopentolate 1% (Alcon). Two 30-gauge needles were introduced through the limbus into the anterior chamber, and 0.7 mL air or SF6 was injected while the aqueous was tapped from the anterior chamber by passive filling of the second needle. The same procedure was performed in both eyes; the only difference was the nature of the injected gas. Wounds were checked for leaks. Betamethasone acetate and phosphate (3 mg in 0.5 mL; Sandoz, Boucherville, QC, Canada), tobramycin (10 mg in 0.25 mL; Alcon), and cefazolin (50 mg in 0.25 mL; Novopharm, Toronto, ON, Canada) were injected in the inferior fornix, and 1 drop of atropine 1% (Alcon) was instilled. Prednisone (5 mg/d; Apotex, Weston, ON, Canada) was given orally on a daily basis.

Postinjection Follow-up

All animals were examined on a daily basis for 10 days after injection. Slit lamp assessment (Haag-Streit, Bern, Switzerland) was allowed to document the decrease in air and SF6 bubble size and the signs of inflammation. The size of the bubble in the anterior chamber was estimated by measuring the ratio between the diameter of the bubble and that of the corneal diameter in the vertical meridian. Aqueous flare and cell responses were graded according to the criteria of Schlaegel et al.8 Intracocular pressure (IOP; Tonovet, TV01; Tiolat Oy, Helsinki, Finland) and central corneal thickness (CCT; Ultrasound Pachymeter SP 3000; Tomey, Nagoya, Japan) were measured on a daily basis. Superior corneal thickness (SCT) and inferior corneal thickness (ICT) measurements and central noncontact specular microscopy (Cellchek XL specular microscope; Konan Medical USA, Torrance, CA) were performed before surgery and 7 and 10 days after the injection. Specular microscopy photographs were taken in triplicate. Eleven animals were euthanatized (pentobarbital sodium 2 mL/4.5 kg IV; Sandoz) on day 10, three animals were euthanatized at 3 weeks and the two animals were euthanatized at 8 weeks. Both eyes were enucleated and examined.

Tissue Preparation

Corneoscleral buttons were dissected and cut in three. A thin, vertical, central, 3-mm-wide band was fixed in glutaraldehyde 2.5% for transmission electron microscopy, and the nasal cornea was fixed in 10% formaldehyde for scanning electron microscopy. Specimens for electron microscopy were then processed as described in Proulx et al.9

The temporal cornea was used for vital staining. Two radial, 4-mm, full-thickness, peripheral relaxing incisions were made to allow full mounting of the specimen. The endothelium was stained with trypan blue 0.25% and alizarin red S 0.2% (Sigma-Aldrich, Oakville, ON, Canada).10 Three standardized photographs were obtained from each of the superior, central, and inferior endothelial areas (objective plan apo; 1.5×; SteREO Discovery V12; Carl Zeiss Canada, Toronto, ON, Canada).

Endothelial Cell Morphometric Analysis

Endothelial cell densities and morphometric analyses of specular microscopy and stereo microscopy images were performed (KSS-409SP software version 2.10; Center Method) available on the Konan (Irvine, CA) noncontact specular microscope system. A minimum of 100 cells were counted in each studied area. The studied parameters included endothelial cell density (cells/mm2) and average endothelial cell area (μm2). The coefficient of variation (CV; SD of cell area/mean cell area) was used as a measure of polymegathism, and the percentage of hexagonal cells was used as an index of pleomorphism.11,12 Bilateral endothelial morphometric analyses were performed. Analyses of the central corneal endothelium as a function of time (before and 7 and 10 days after injection) were based on in vivo specular microscopy. Because only central measurements can be taken with specular microscopy in the living animal, characterization of the geographic distribution of the endothelial cell damage across the corneal surface (superior, central, and inferior cornea) was made using vital staining of postmortem tissues. Alizarin red and trypan blue endothelial stainings were performed on all postmortem corneas. Enucleation was performed at 10 days in 11 animals, 3 weeks in 3 animals, and 8 weeks in 2 animals. Enucleation at day 10 was preferred to detect the early distribution pattern of endothelial damage before cell migration and uniformization of the endothelial mosaic.13-15 The decision to postpone enucleation in five animals was based on the presence of a fibrin membrane attached to the corneal endothelium, either bilateral (four animals) or unilateral (in the SF6 eye of one animal). These membranes might have masked the endothelial mosaic and prevented postmortem morphometric analysis using a posterior approach. The five pairs of corneas with a longer follow-up were first compared with the 11 pairs with a 10-day follow-up. Because cell damage distribution was the same, all corneas were analyzed together.

Statistical Analysis

We studied the effect of two factors (time and type of treatment) on the evolution of the measured parameters (e.g., bubble size, anterior chamber cells, flare). The two treatment modalities assessed were air and SF6. The effect of time was measured from day 0 to day 10 after injection. ANOVA with two repeated factors was used on full data sets. In case of an interaction between the type of treatment and time, each of these two factors was analyzed separately with repeated-measures ANOVA or paired t-tests while fixing the other factor. Mean and SEM are reported. Analyses were performed with SPSS (Chicago, IL) software, version 15.0, and P < 0.05 was considered statistically significant.

RESULTS

Bubble Size

Bubble size was studied as a function of time after injection and type of treatment. A significant statistical interaction was found between time and type of treatment (P < 0.001), and both parameters were found to affect bubble size. On the day of injection (day 0), the bubble size was the same in both groups (air and SF6), but it decreased significantly more quickly in the group injected with air (Figs. 1A–F, 2A). Complete clearance of the bubble in all eyes took 9 days with air (0.50% ± 0.35% of the anterior chamber volume at day 8 and no air at day 9) and >10 days with SF6 (2.19% ± 1.29% of the anterior chamber volume at day 10).

Anterior Chamber Inflammation

A significant statistical interaction was found between time and type of treatment for each of the two parameters of anterior chamber inflammation (cells and flare; P < 0.001). Air and SF6 induced inflammation in both groups (Figs. 2B, 2C). This inflammation was significant from day 1 (P < 0.001 for cells and flare), reached a maximum on day 2, and decreased progressively. The amounts of cells and flare were greater and decreased significantly more slowly with SF6 than with air. Inflammation did not subside totally by day 10 (mean difference in cell grade between presurgery and day 10: air, 1.19 ± 0.23, P = 0.006; SF6, 1.69 ± 0.25, P < 0.001; mean difference in flare: air, 1.50 ± 0.30, P = 0.010; SF6, 2.25 ± 0.35, P = 0.001). In some cases, inflammation led to...
the formation of a loose fibrinous membrane in the anterior chamber, which tended to wrap the bubble and attach either to the iris surface or to the cornea.

**Intraocular Pressure**

No statistical interaction was found between time and type of treatment. Intraocular pressure was affected by time after injection \((P < 0.001)\) but not by type of treatment (air vs. SF6). In both groups, a temporary and nonsignificant increase in IOP was seen early after injection (mean increase, \(3.2 \text{ mm Hg}; P = 0.255\); Fig. 2D). IOP then decreased below preoperative levels (this decrease was significant from day 5) and remained low until the end of the 10-day study period.

**Corneal Thickness**

Analysis of corneal thickness was limited by the high proportion of missing data because ultrasound pachymetry could not be performed in the presence of a retrocorneal bubble in direct contact with the cornea. Corneal thickness was measurable in all eyes before surgery. In eyes injected with air, time after injection and position on the cornea (SCT, CCT, ICT) had no significant effect on the ability to measure corneal thickness. In eyes injected with SF6, the percentage of available measurements was significantly affected by time and position on the cornea (on day 10, the percentages of available data were still inferior to normal: SCT, 25%; CCT, 56%; ICT, 94%; SCT, <ICT; \(P < 0.001\)).

**Central Corneal Thickness.** As a consequence, analyses on complete CCT data sets (air and SF6 from day 0 to day 10) could only be performed on four animals (Fig. 2E). No interaction was found between time and type of treatment. CCT was affected by type of treatment (central corneas were thicker with SF6 than with air; \(P = 0.019\)) but not by time of treatment. Corneas exposed to SF6 remained thicker until the end of the study period.

**Inferior and Superior Corneal Thickness.** Analyses on complete ICT data sets (air and SF6 from days 0, 7, and 10) could be performed on 13 animals. No significant interaction was found between time and type of treatment. The ICT was not affected by type of treatment, but it was affected by time after injection \((P = 0.002)\). Paired comparisons revealed a significant postinjection increase in ICT (ICT: preinjection, \(684 \pm 18 \mu m\); day 7, \(732 \pm 20 \mu m \ [P = 0.007]\); day 10, \(724 \pm 21 \mu m \ [P = 0.046]\)). Statistical analyses could not be performed on SCT because of the high number of incomplete data sets.

**Specular Microscopy**

**Endothelial Cell Counts.** A significant interaction was found between time and type of treatment \((P = 0.025)\). Although cell counts were similar in both groups before injection, they were significantly lower after injection in the SF6 group (mean air-SF6 difference before injection: \(-35 \pm 37 \text{ cells/mm}^2, P = 0.357\); after injection: \(128 \pm 41 \text{ cells/mm}^2, P = 0.008\)). Postinjection values consisted in the mean of day 7 and day 10 values. A mean cell loss of \(132 \pm 50 \text{ cells/mm}^2\) was observed in the SF6 group \((P = 0.021\); Fig. 3A).

**Average Cell Area.** Similarly, a significant interaction was found between time and type of treatment \((P = 0.034)\). Al-
though the average cell area was similar in both groups before injection, cells were significantly larger after injection in the SF6 group (mean air-SF6 difference: preinjection, 25.86 ± 6.12 μm², P = 0.049; postinjection, −24.85 ± 8.72 μm², P = 0.014; Fig. 3B). SF6 induced a significant increase in cell area, but air did not (mean increase: SF6, 25.52 ± 10.01 μm², P = 0.024; air, −4.10 ± 5.56 μm², P = 0.473).

Coefficient of Variation of Cell Area. No interaction was found between time and type of treatment. The CV increased after injection in both treatment groups (preinjection, 24.05 ± 0.68; postinjection, 28.58 ± 1.11; P < 0.001; Fig. 3C). The CV was also globally higher in the SF6 group (air, 25.86 ± 0.67; SF6, 28.03 ± 1.14; P = 0.007). However, because this difference was also present before injection, no conclusion could be drawn regarding the comparative effect of air and SF6 on the CV.

Hexagonality. Once again, no interaction was found between time and type of treatment. Hexagonality was affected only by time; both treatments induced a similar decrease in the percentage of hexagonal cells (before injection, 67.01% ± 1.06%; after injection, 61.87% ± 1.43%; P = 0.009; Fig. 3D).

Alizarin Red and Trypan Blue Staining
Morphometric analyses demonstrated that signs of endothelial cell damage and instability were highly dependent on the position on the cornea (superior, central, or inferior), with a greater damage in the superior position. Figures 1G to 1L illustrate the typical aspect of the endothelium after injection of air in one eye (third column) and SF6 in the contralateral eye (fourth column).

Endothelial Cell Density. A significant interaction was found between position and type of treatment (P = 0.045). Although position on the cornea did not affect endothelial cell density in eyes injected with air, endothelial cell loss was significantly greater in the superior cornea of eyes injected with SF6 (mean difference in cell density with SF6: superior-center = −615 ± 186 cells/mm², P = 0.016; superior-inferior = −1224 ± 290 cells/mm², P = 0.003; center-inferior = −609 ± 189 cells/mm², P = 0.018; Fig. 4A). In the superior position, however, the overall difference between the two groups only tended to be significant (mean air-SF6 difference: 619 ± 303 cells/mm², P = 0.060).

Average Endothelial Cell Area. No interaction was found between position and type of treatment, and neither position nor type of treatment was found to affect the average cell area (Fig. 4B).

Coefficient of Variation of Cell Area. No interaction was found between position and type of treatment. A significant position effect was observed (P = 0.011), but no differences were detected between air and SF6 groups (Fig. 4C).
differences were statistically significant (superior-center/highly dependent on corneal position). The percentage of hexagonality was increased endothelial cell area (Fig. 5A–D). Inflammatory cells consisting of cell membrane disruption, missing cells, and exposed to air, and a greater damage in the superior cornea, were more viable. An example of the transmission electron mi-

Transmission Electron Microscopy
In corneas exposed to SF6, it was found that the endothelial cells were thinner and more elongated than in the corneas exposed to air. Some endothelial cells appeared to be detached from Descemet’s membrane and showed nonspecific protein accumulation in the subendothelial cell space. A definite disturbance in the structure of the mitochondrial cristae was observed and was a sign of cell stress. The inflammatory membrane seen over the endothelium included multiple cells, among which monocytes, immature fibroblasts, and epithelioid histiocytes (ker-atomic precipitates) could be identified. Some similar but less advanced and more subtle changes were observed in the endothelial cells of corneas exposed to air. It could be seen that these cells were more viable. An example of the transmission electron mi-

Scanning Electron Microscopy
Scanning electron microscopy was performed on four representative pairs of eyes. It confirmed the observations made by specular microscopy and vital staining, namely a greater endothelial cell damage in eyes exposed to SF6 than in those exposed to air, and a greater damage in the superior cornea, consisting of cell membrane disruption, missing cells, and increased endothelial cell area (Fig. 5A–D). Inflammatory cells scattered on the posterior surface of the cornea were occasionally found in eyes exposed to either air or SF6 (Fig. 5E).
crosscopy aspect of the endothelium of a pair of corneas exposed to air and SF6 is shown in Figure 6.

**DISCUSSION**

Our results demonstrate that in the living feline model, both air and SF6 injected into the anterior chamber of the eye can induce an intraocular reaction. SF6 was more toxic than air in terms of anterior chamber inflammation (cell, flare, and corneal edema) and corneal endothelial cell losses, and the endothelial damage was significantly greater in the superior cornea. Air did not significantly affect central endothelial cell counts, and it had no effect on cell damage distribution.

Both air and SF6 induced pleomorphism and polymegathism, two signs of reversible endothelial instability, which herein were primarily marked in the superior cornea.

To the best of our knowledge, no previous report on the corneal endothelial toxicity of intracameral gases16–21 compared air and SF6 toxicity in a living animal model with corneal endothelial wound healing characteristics similar to those of human subjects. The rabbit corneal endothelium model, which was used most often,16–20 is known to differ significantly from the human model. Rabbit endothelial cells actively regenerate after wounding, showing extensive cellular division at the margin of the wound, migration, elongation, coalescence, endothelial multilayering, and replacement of old degenerated endothelial cells with new endothelial cells.15,16,22,23 Endothelial repair in the rabbit is rapid and occurs in hours or days, depending on the severity of the damage.15,25 Contrary to the rabbit, endothelium repair in the human takes place without mitosis.24–26 Cells enlarge, spread, and migrate to cover the deficit left by the loss of neighboring cells, eventually restoring a more stable pattern but never returning to a normal size, and the cell count remains low.27 The nonregenerative properties of the corneal endothelium of the cat were shown to be similar to those of the human corneal endothelium.13–15,22

**FIGURE 6.** Transmission electron microscopy. Pair of corneas of the same animal exposed to SF6 in one eye (A) and air in the other eye (B). In the cornea exposed to SF6, a nonspecific material lay in the subendothelial cell space. An inflammatory membrane, in which multiple cells such as monocytes, immature fibroblasts, and epitheloid histiocytes (keratic precipitates) were identified, covered the endothelium. No inflammatory membrane was found over the endothelial cells of cornea exposed to air. Scale bar: 2 μm (A, B).
Several older reports using varying methodologies, mostly descriptive, occasionally quantitative, and usually in the rabbit model, all suggest some degree of corneal endothelial toxicity (corneal edema, aqueous flare and fibrin, and endothelial cell loss) for both air and expansive gases. Studies from Olson et al. and Fouklks et al. report results in the cat model in accordance with our findings, showing signs of intraocular toxicity to air and SF6, respectively.

As mentioned by Green et al. and as suspected herein, longevity of the gas in the anterior chamber seems to play a key role in the severity of its deleterious effect. In the present study, the endothelial cell damage was located primarily in the superior cornea, where the gas bubble remained for the longest period. This finding parallels results from Doi et al. showing increased retinal toxicity (abnormal glutamate distribution and thinning of the outer plexiform layer) of intravitreal injection of air, SF6, and C3F8 in the superior retina, where gases were in continuous contact.

Endothelial toxicity may be explained by several mechanisms. Interference of the bubble with the aqueous humor nutrients would most likely be limited to cases in which the bubble fills the entire anterior chamber, with smaller mobile bubbles allowing enough exchange between the endothelium and the aqueous humor.

A mechanical interaction, resulting either from surface tension or from direct trauma by the bubble itself, could be a possible source of endothelial cell damage. Hong et al. used corneas mounted on an artificial anterior chamber filled at 40% with an air bubble and rotated 180° for 50 times to simulate the movement of an eye filled with air after DSAEK. The proportion of viable endothelial cells documented by trypan blue and alizarin red staining was significantly lower after air bubble trauma (79.8% ± 0.04%) than was the proportion of control fellow corneas (89.9% ± 0.02%; P = 0.05; n = 12 pairs). In the present study, the surgical stress was the same for all eyes and cannot be held responsible for differences between groups.

Endothelial cell injury could also be secondary to inflammation, which in our study was more severe with SF6 than with air. An inflammatory reaction in cat and rabbit eyes after the intracameral injection of air, SF6, or C3F8 has been reported by others. The inflammatory response observed herein in cat eyes was more marked than that routinely observed in human eyes, such as after DSAEK surgery.

Changes in the antioxidant system of the aqueous humor have also been reported after intravitreal injection of SF6, as measured by the increased activity of catalase and superoxide dismutase and increased malondialdehyde concentrations. These authors concluded that the increased occurrence of active oxygen species in the aqueous humor leads to insufficiency of the antioxidant system and intensification of the peroxidation processes, reflected by increased malondialdehyde concentration.

It should be mentioned that the numerous reports on the beneficial effect of air injected into the anterior chamber to prevent its collapse during intracapsular or extracapsular cataract extraction were of no help in the present study. In these older reports, the considerable mechanical protection offered by the short-term (few minutes only) filling of the anterior chamber with air would have masked any eventual sign of air endothelial toxicity.

In conclusion, both air and SF6 injected into the anterior chamber of the eye were shown to induce intraocular reaction in the living feline model. SF6 was more toxic than air in terms of endothelial cell loss and anterior chamber inflammation. Based on the results obtained herein, the lack of endothelial toxicity of air and SF6 in the human subject should not be considered as granted. We therefore recommend that these gases be used with caution in the anterior chambers of human subjects. Their use should be at the minimal dose necessary to obtain the desired tamponade effect and for no longer than required, and air should be favored over SF6.

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