Experimental corneal calcification in animals treated with dihydrotachysterol

**Jiří Obenberger,** * David E. Ocumpaugh,** and Margaret G. Cubberly

Several methods of producing calcification of the cornea in experimental animals are presented. Experimental corneal calcification resulted from a combination of treatment with dihydrotachysterol (DHT) and corneal injury. In rabbits, six varieties of corneal injuries were inflicted: de-epithelization of the entire or central part of the cornea, abrasion of the central posterior surface, a combination of these procedures, freezing, and intracameral injection of potassium permanganate. Freezing the central cornea was the simplest and most effective process for the rabbit. In the rat, de-epithelization of either the entire or central cornea proved most effective of the methods employed. In both species, calcification occurred within one week and was always found just beneath the corneal epithelium. Treatment with DHT proved necessary for mineralization, no matter what the operational procedure. The presence of calcium deposits in tissue sections was demonstrated by von Kossa's histochemical method and ⁴⁴Ca autoradiography. Moderate calcification persisted for many months. Larger subepithelial plaques provoked vascularization and rapid disappearance of the calcium deposits. The procedures employed are discussed in terms of their possible usefulness in future studies of corneal and other soft tissue calcification. A possible role of fibroblasts is suggested.

**Key words:** corneal calcification, dihydrotachysterol, corneal injuries, corneal freezing, potassium permanganate, injections, corneal epithelium, corneal stroma, corneal endothelium, fibroblast, corneal vascularization, slit lamp examination, guinea pigs, rabbits, rats.

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Experiments designed to produce calcification in the cornea have not, to our knowledge, been previously reported. However, corneal calcification has been inadvertently elicited in a variety of laboratory situations. Calcification in the cornea was noted in rabbits after ligation of the vortex veins, in newborn rabbits with congenital cataract, after exposure of the cornea to lime and the cations yttrium, lanthanum, samarium, and gadolinium, and in rabbits used for uveitis experiments.
and accidentally affected by hypervitaminosis D. Deposits of calcium in the rat cornea were seen after combined treatment with isoproterenol, dihydrotachysterol, and 5-hydroxytryptamine and, more recently, after the application of morphine sulfate, where the main factor seemed to be drying of the cornea.

In this experiment, a simple method of producing calcification in the cornea was sought to provide a model for the study of a common disease of man. The authors believed such a model would prove useful also in the study of the more general phenomenon of soft tissue calcification.

Selye and co-workers were successful in eliciting calcification in the skin of rats by systemic administration of dihydrotachysterol combined with cutaneous injury. Because of this success, it was decided, in the current work, to attempt several variations on their approach in an effort to produce calcification in the cornea of the rat, guinea pig, and rabbit.

Materials and methods

Sherman strain rats weighing 80 to 200 grams, albino and pigmented rabbits weighing 2.5 kilograms, and random-bred albino and pigmented guinea pigs of 250 grams were used. Animals were unselected as to sex, and were fed standard diet pellets (Agway, Inc., Syracuse, N. Y., for guinea pigs and rabbits, and Ralston Purina Company, St. Louis, Mo., for rats).

Dihydrotachysterol (DHT) (Mann Research Labs., N. Y.) was dissolved in corn oil (2.0 mg. per 1.0 ml.), and 1 mg. of DHT per 100 or 1,000 Gm. of body weight was administered by stomach tube to rats and rabbits, respectively. Variations from this dosage were made to determine maximal and minimal effects. Typically, DHT was given 24 hours before corneal injury. In some cases, it was administered the same day, or as much as 48 hours before or after corneal damage was inflicted. DHT was given to guinea pigs intramuscularly in an average dose of 0.5 mg. per 100 Gm. of body weight.

Corneal injury. Six kinds of corneal injury were performed: (1) de-epithelization of the entire cornea of rat, guinea pig, and rabbit with a knife; (2) de-epithelization of an area 3 mm. and 6 mm. in diameter (rats and rabbits, respectively) with a knife; (3) abrasion of a central area of the rabbit endothelium and Descemet's membrane 2 to 3 mm. in diameter with a curette; and the posterior layers of rat and guinea pig corneas with a bent 27 gauge needle; (4) combination of operations 1 and 3 with the rabbit; (5) intracameral injection of rabbits with potassium permanganate (0.4 ml. of 0.1 per cent aqueous solution); and (6) freezing of rat and rabbit corneas with instruments cooled with dry ice and alcohol (−72° C.). Instrument tips were curved to fit corneas of both species. The procedure was identical to that introduced by Dunington and Smelser.

All operations were performed on animals anesthetized with Nembutal and with topical instillation of proparacaine hydrochloride (Ophthaine, Squibb and Sons, N. Y.). Neosporin ophthalmic solution (Burroughs Wellcome and Company, N. Y.) was administered locally after the operation. During the first postoperative week, the animals were examined daily with the slit lamp. In subjects where calcification was observed, the corneas were checked weekly. Selected cases were photographed and studied histologically.

Histologic methods. The anterior segments of all the eyes were fixed for 12 to 16 hours in a solution of 1 part 10 per cent neutralized formalin and 4 parts absolute alcohol. The tissues were then dehydrated, embedded in paraffin, and sectioned at 4µ. Most sections were stained with hematoxylin and eosin and by von Kossa's method for calcium demonstration. Some sections were stained by other methods for the histochemical detection of calcium, such as chloranilic acid, celestin blue, alizarin red S, murexide, sodium rhodizionate, and naphthachrome green B.

Autoradiography. An injection of 45Ca (specific activity 160 µC per milliliter in isotonic saline) was given intraperitontially to three albino rats as well as intracameraly to nine albino rabbits.

The three rats were treated with DHT and their corneas de-epithelialized the same day. The animals (body weight 140 grams) were injected with 130 µC of radioactive calcium at 1, 2, and 6 days after surgery. Each animal was put to death 5 hours after injection.

Four rabbits were treated with DHT, and 24 hours later the right cornea of each animal was frozen and the left cornea de-epithelialized. Eight µC of 45Ca was injected into the anterior chamber of each eye. The four rabbits were injected 2, 3, 6, and 7 days, respectively, after the operation. Three additional rabbits were operated on as above. DHT was omitted in these, and the animals were injected 2, 3, and 6 days after the operation. Finally, the two remaining rabbits were neither given DHT nor operated on, and received only 45Ca injections into the anterior chamber of both eyes. All nine rabbits were...
killed 24 hours after administration of radioactive calcium.

The corneas were processed as described above. Tissues were sectioned at 4 μ thickness. Some sections were stained by von Kossa's method for detection of calcium. Alternate sections were prepared for autoradiography by the dipping technique with Ilford G 5 liquid emulsion. The slides were exposed for 1 to 6 weeks under the emulsion at 4° C. at low humidity, developed in Kodak D 19 for 5 minutes, fixed in NH-5 (Heico, Inc.), and stained with hematoxylin.

Results

Table I summarizes results with respect to the type of operation employed. Clinical signs of calcification were produced in varying degree in both rats and rabbits with every type of operation, when combined with DHT treatment. No mineralization took place when DHT was omitted. In rats, the most effective method was de-epithelization of either the central or entire cornea. In rabbits, the most successful method was freezing of all the corneal layers. Calcification was obtained in only 2 of 16 guinea pigs. Therefore, the results are not tabulated here. Calcification always occurred in the stromal layers just beneath the epithelium; moreover, it was always observed in regions where there was an abundant population of fibroblasts.

In rats weighing more than 120 grams, the routine dose of 1 mg. per 100 Gm. of body weight resulted in death in only 10 per cent of the animals. In rabbits, the average dose was one tenth that used in rats, i.e., 1 mg. per 1,000 Gm. of body weight. Greater dosages produced greater intensity of calcification, but also more premature toxic deaths. The lowest effective dose required to induce calcification in rabbits was 0.25 Gm. per 1,000 Gm. of body weight. No correlation was found between the time of DHT treatment and corneal injury, and the clinically observed sequelae if DHT was given within 48 hours.

The results of the different methods employed with both rats and rabbits are described below:

1. De-epithelization of the whole cornea was one of the most effective procedures for rats (Table I). For three days after the operation and DHT treatment, the corneas showed an edema which subsided with reepithelization. The first signs of calcification, small white granules just beneath the epithelium, were seen under the slit lamp at this time. After seven days, the cornea

<table>
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<th>Animal</th>
<th>Type of trauma</th>
<th>Number of corneas</th>
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<tbody>
<tr>
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<td>Calcification observed</td>
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<td>Rat</td>
<td>1. De-epithelization of whole cornea</td>
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<td>2. De-epithelization of central cornea</td>
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<td>3. Abrasion of the posterior surface</td>
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<td>4. Freezing of the cornea</td>
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<td></td>
<td>Unoperated control corneas</td>
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<tr>
<td>Rabbit</td>
<td>1. De-epithelization of whole cornea</td>
<td>5</td>
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<tr>
<td></td>
<td>2. De-epithelization of central cornea</td>
<td>2</td>
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<tr>
<td></td>
<td>3. Abrasion of the posterior surface</td>
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<td>4. Nos. 1 + 3 combined</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5. Permanganate injection</td>
<td>11</td>
</tr>
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<td></td>
<td>6. Corneal freezing</td>
<td>47</td>
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<td></td>
<td>Unoperated control corneas</td>
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*All animals were observed for 6 months after operation. An additional group of 32 rats and 39 rabbits, not included here, were put to death before clinical signs of calcification were evident in order to study early histological changes.
Fig. 1. Schematic drawing, as seen with slit lamp, showing varieties of calcification after de-epithelization of the cornea in rats pretreated with DHT. A, Tiny subepithelial whitish deposits which form a "mesh"; B, round-shaped subepithelial opacity localized predominantly in nasal half of cornea; C, calcified plaque invaded by blood vessels.

Fig. 2. Calcium deposits (arrow) between the epithelium and stroma of an otherwise normal rat cornea. The cornea was de-epithelized 24 hours before treatment with DHT. (von Kossa's method, Harris hematoxylin; ×600.)

Fig. 3. Radiograph showing subepithelial deposits of 45Ca in a rat cornea (arrow). The corneal epithelium had been removed and the rat treated with DHT on the same day. Radioactive calcium was injected on the sixth day after corneal injury. (Harris hematoxylin; ×300.)

was completely re-epithelialized and the subepithelial calcium deposits were more extensively developed, being visible with the naked eye. There was a regional predilection for the nasal portion (Fig. 1, A). The presence of calcium in these areas was determined by von Kossa's staining technique (Fig. 2), and the specificity of this method was confirmed by autoradiography with 45Ca (Fig. 3).

In some instances, the calcium precipitates were more extensive and less dense (Fig. 1, B). These deposits persisted for many months, diminishing only gradually. However, where calcium deposits were more abundant, an inflammatory reaction,
including invasion of blood vessels, occurred. Such heavy deposits disappeared rapidly from the cornea (Fig. 1, C).

2. De-epithelization of the central stroma of the rat cornea resulted in a process of calcification similar to the events described above. In nearly all rabbits, only normal re-epithelization without calcification was seen (Table I).

3. Scraping of the endothelium and Descemet's membrane of rabbit cornea resulted in a series of events similar to those described above. Edema occurred soon after the operation and white subepithelial spots appeared by the third to sixth days, gradually growing more extensive. The peripheral, moderately edematous, area of the corneal wound was much less frequently affected by calcifications than the central one (Fig. 4). These deposits remained over half a year with only slight decrease in the extent of calcification. Similar treatment of rat corneas was relatively ineffective.

4. De-epithelization of the rabbit cornea, followed by abrasion of the posterior surface, produced the same type of calcification as described above.

5. Intracameral injection of permanganate produced calcification in 84 per cent of the corneas treated. Calcification often involved nearly the entire subepithelial surface of the cornea and sometimes extended deep into the anterior stroma (Fig. 5). Deposits were formed as rapidly as in the other methods employed. The extent of the development, both in surface area and in depth, however, was highly variable.

6. Freezing of the cornea was the most consistent and predictable mode of corneal injury in the rabbit: 94 per cent of the corneas developed calcification (Table I). The diameter of the subepithelial calcium plaque which developed was determined (approximately) by the diameter of the freezing instrument employed. The sequence of events was similar to that described previously. Minute white subepithelial granules appeared 3 or 4 days after the operation. Calcium deposits thickened and became easily visible by the sixth day (Fig. 6). As in other types of corneal calcification, development of further changes seemed to depend on the amount of precipitate. When deposits became heavy, blood vessels invaded and encircled the plaque. The calcified mass became broken up and rapidly disappeared (Fig. 7).

Most cells in the frozen region were killed at the outset. New fibroblasts migrated into the area. Calcification was detected only by histochemical and auto-
Fig. 6. A calcium plaque is seen developing in the rabbit cornea just below the level of the pupil. Sixth day after DHT treatment and freezing of the cornea.

Fig. 7. Schematic drawing on the forty-second day after freezing of the rabbit cornea. The central cornea is occupied by several pieces of chalky-white, broken plaques. Capillary nets, originating from several larger vessels, appear to be invading the calcified precipitate.

Fig. 8. Calcium deposits bordering epithelium and in spaces occupied by stromal cells in rabbit cornea. Seven days after DHT treatment and six days after corneal freezing. (Celestin blue; x800.)

Fig. 9. More advanced stage of calcification in subepithelium of rabbit. Animal treated with DHT and subsequent corneal freezing. (von Kossa's method, neutral red counterstain; x800.)

Radiographic means in the subepithelial region where new fibroblasts were present. Calcification was always absent in stromal areas where such cells were absent.

In histological sections stained with the von Kossa and celestin blue techniques, it appeared that some calcium granules impregnated collagen bundles, and other deposits were concentrated in the fibroblasts (Figs. 8 and 9). Autoradiography showed the same distribution of calcium deposits subepithelially as was demonstrated by the von Kossa stain (Figs. 10 and 11). Consistently, von Kossa's technique for detecting calcium more closely paralleled the findings with $^{45}$Ca autoradiograms than did the other histochemical stains.

Discussion

In rabbits, the simplest and most repeatable procedure for inducing calcification in the cornea was freezing of the corneal layers. In 3 to 6 days, 94 per cent of the frozen corneas developed calcification. The diameter of the subsequent plaque was determined by the diameter of the freezing tool employed. Such a procedure may prove useful in the study of soft tissue calcification. Because the cornea is a transparent tissue, calcification can be followed from its earliest beginning. With the rabbit, various chemicals, including drugs and isotopes, may be easily administered to the corneal surface, subconjunctivally or intra-camerally. Another advantage of corneal...
Fig. 10. Restriction of calcification to the superficial stroma of rabbit is shown where the corneal epithelium is detached. Seventh day after freezing and DHT treatment. (von Kossa's technique; ×150.)

Fig. 11. A radioautograph of same cornea as in Fig. 10. Animal was injected with $^{45}$Ca 24 hours prior to being put to death. Incorporation of the isotope is limited to the superficial stroma. It appears that distribution of calcium, as demonstrated by this radioautographic technique, is markedly similar to that shown by von Kossa's staining. (×150.)

tissue is that it is available in a simple form, i.e., free of vascular elements or subcutaneous tissue, for further biochemical or histochemical analysis. It was seen that vascularization and complex inflammatory reactions never occurred during the calcifying process, except when very large plaques were formed. Finally, the contralateral cornea provides the best possible experimental control.

While de-epithelization plus DHT treatment was effective in producing corneal calcification in the rat, this species will probably be less useful than the rabbit for the application of experimental procedures. Due to the small size of the rat eye, it is more difficult to administer chemicals intracameraly and subconjunctivally, or to damage (or otherwise treat) very restricted areas of the corneal tissue.

The relative failure to provoke corneal calcification in guinea pigs may be ascribed either to insufficient treatment with DHT, or resistance of these animals to the action of DHT, or both.

In all six procedures reported here, calcification was preceded by edema. DHT administration, which elevate blood calcium levels, proved necessary in every case. Moreover, calcification was always confined initially to the subepithelial stroma, where there were abundant fibroblasts. This phenomenon was most strikingly demonstrated by the freezing method. Most cells in the frozen area were killed. New fibroblasts migrated into the devitalized area and became concentrated beneath the epithelium. Calcification subsequently began in this region; calcification never occurred in subepithelial or other stromal areas where such cells were absent. Studies in progress indicate that these subepithelial fibroblasts are more actively synthesizing mucopolysaccharides and proteins than the remaining stromal cells. Since the onset of calcification appears associated with this activity, it seems that these fibroblasts may be participating in the formation of the calcium-binding material. It is not yet known why there is
a consistently large concentration of active fibroblasts just beneath the epithelium during wound repair in the cornea. Dunnington and Smelser\(^9\) have speculated that this high cellular activity may be due to the greater nutritive environment provided by the epithelium (which is glycogen and oxygen rich) than by the milieu of the deeper stroma.

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**REFERENCES**