Reciprocal Corneal Transplantation Fails to Correct Mucopolysaccharidosis VI Corneal Storage

Gustavo Aguirre,*† Irv Rober,*‡ Myron Yanoff,*§ and Mark Haskins†

This report contains the results of studies designed to evaluate corneal clearing in mucopolysaccharidosis VI (MPS VI)-affected cats. Corneal buttons from affected cats were transplanted into normal cat corneas and, as controls, normal-to-normal and normal-to-affected transplants also were done. No clearing of the MPS VI graft or host beds occurred, nor was there any clouding of the normal donor or recipient corneal tissues. This assessment was made by serial clinical examinations over a 14-30 mo period and by light and electron microscopic examination of the corneal tissues at the end of the study. Lack of corneal clearing under conditions that would maximize such a process in this animal model indicates that corneal clearing is not an appropriate index for measuring the success of systemic therapy in MPS VI.


The mucopolysaccharidoses (MPS) represent a heterogeneous group of inherited disorders in humans, characterized clinically by skeletal deformities and facial dysmorphia, central nervous system or visceral storage, and, in most, corneal clouding. Each clinical entity represents the deficiency in activity of a specific lysosomal enzyme involved in the degradation of the acidic mucopolysaccharide (glycosaminoglycan, GAG) component of extracellular matrix proteoglycans. This results in the intracellular accumulation of undegraded substrates (heparan, dermatan, keratan or chondroitin sulfates) in secondary lysosomes. The affected cells enlarge, their function is compromised, and the characteristic clinicopathologic features of the disease evolve. Animal models for these diseases have been reported in the cat (MPS I, MPS VI), dog (MPS I, MPS VII), and mouse (MPS VII), and the biochemical defects and ocular disease have been reviewed.

After the in vitro demonstration that soluble "corrective factors" are released into the media and reverse substrate storage, as determined by the reduction of 35S label in diseased cells, various therapeutic strategies were attempted in the MPS storage disorders. Therapy has been directed at restoring the deficient enzyme function by enhancing residual activity and by providing normal enzyme directly or indirectly by transplantation of bone marrow or other cells. Because the clinical disease may be variable, and most patients show the disease phenotype at the time therapy is initiated, establishing criteria by which the success of therapy can be evaluated had been difficult. Determination of enzyme activity in the serum or leukocytes provides an objective measurement, but one that may not be appropriate because the assays are made using artificial rather than natural substrates, and the presence of normal activity in the blood does not necessarily mean that normal degradative function is occurring in the lysosomal compartment of the other affected tissues. More objective criteria, using noninvasive methods—for example, electroretinographic assessment of retinal function, radiographic evaluation of skeletal/articular disease, quantitation of urinary GAGs, or determination of liver size by magnetic resonance imaging—have the advantage of being repeatable and can be used with equal validity in human patients as well as in experimental animal models.

Corneal clouding is a characteristic feature of most of the MPS disorders. In some human patients after bone marrow transplantation, the degree of corneal clouding, evaluated directly or indirectly by visual acuity determination, has been reported to decrease in parallel with an improvement in the patients' overall condition. In one patient, however, although visual acuity improved after bone marrow transplantation, no clearing of the cornea occurred.
MPS VI patients who underwent successful corneal transplantation to correct the corneal clouding, there was clearing of the recipient cornea directed, presumably, by the normal graft. In the MPS-affected animal models (MPS VI cat and MPS I dog), a decrease in corneal clouding also has been reported after successful bone marrow transplantation. However, a re-evaluation of corneal clouding in MPS VI affected cats has shown little if any clearing of the cornea after transplantation.

The clearing of the cornea in human and animal patients with MPS suggests that normal enzyme diffuses into the avascular cornea from the limbus or, as in Naumann’s case, from the normal graft to the deficient recipient cornea, to correct the metabolic defect and restore normal corneal clarity. These results also suggest that the evaluation of corneal clearing could be a useful way to noninvasively monitor the response to therapy after bone marrow transplantation. To test this hypothesis, we performed a series of studies in which corneas from MPS VI-affected cats were transplanted into normal hosts, and in which the status of the cornea was monitored by clinical and histopathologic examinations. The results show that no significant clearing of the transplanted corneas occurred during the 3 yr postoperative period of observation.

Materials and Methods

Animals

The normal and MPS VI-affected cats used in this study were part of a research colony developed and maintained at the School of Veterinary Medicine, University of Pennsylvania. The affected animals were identified by their characteristic clinical phenotype and deficient arylsulfatase B (ASB) activity in peripheral blood leukocytes or cultured fibroblasts, or both. The normal ASB status of the animals used as donors or recipients for the corneal transplant also was established on the basis of normal ASB activity in peripheral blood leukocytes. The animals were raised and maintained in standard animal care facilities with cyclic illumination, in full compliance with the NIH Guidelines and the ARVO Resolution on the Use of Animals in Research.

Corneal Transplant Surgery

The three different transplantation paradigms used the same surgical technique: normal donor to normal recipient (two eyes), normal donor to MPS VI-affected recipient (two eyes), and MPS VI-affected donor to normal recipient (three eyes). Specific details on the animals studied and the post-transplantation observation period are presented in Table 1. The surgical procedure and immediate postoperative care used was that described experimentally for cats by Bahn and associates with the following modifications. McCarey-Kaufman medium was used for temporary storage of donor buttons (1-3 hr) prior to transplantation, and general anesthesia (intravenous thiobarbiturate for induction, Forane [Anaquest, Madison, WI] inhalation for maintenance) was used and supplemented with pancuronium to facilitate eye positioning. Postoperatively, some of the animals had mild to moderate corneal vascularization, which was controlled by topical steroid drops—with (Maxitrol; Alcon, Ft. Worth, TX) or without (Pred Forte; Allergan, Irvine, CA) antibiotics—used 1-3 times per day until sutures were removed (2-4 wk). Treatment was gradually discontinued thereafter.

Clinical and Pathologic Evaluation

Clinical examinations were performed on all of the experimental animals at regular time points after transplantation. Examinations were made with a slit-lamp microscope or hand held fundus camera, or both, in the awake animal. Because corneal clarity was evaluated most readily by retroillumination, the latter instrument was found to be especially useful in the awake animal. At selected intervals and for the final examination, the animals were anesthetized with intramuscular ketamine, and high resolution biomicroscopic examination of the cornea, with or without photography, was carried out. The length of postoperative follow up, including the number of observations, are detailed in Table 1. MPS VI-affected cats raised and housed in the same animal colony were used as controls for the corneal storage. They provided a basis upon which the clinical evaluation of corneal clarity was made.

At the end of the study, the cats were anesthetized with intramuscular ketamine and killed with an overdose of pentobarbital sodium administered by the intravenous or intracardiac routes. Immediately after the animals' deaths, the eyes were enucleated, and the anterior segments were removed with scissors after an incision was made in the sclera just behind the limbus. The corneoscleral tissues, including the lens, were placed in 3% glutaraldehyde/2% formaldehyde in cacodylate buffer. While in this fixative, the lens was removed by a zonulotomy, and the cornea was trimmed into quarters whose apices were at the center of the graft. Opposing quarters were processed further for plastic embedding for light and electron microscopic examination by continuing the fixation in 2% glutaraldehyde/1% osmium tetroxide, and 2% osmium tetroxide. Details of this triple fixation protocol have been described. After dehydration, the speci-
Table 1. Summary of the experimental animals and procedures

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* Age at transplant.
† Size of graft button in millimeters vs. size of recipient bed.
‡ Examinations made after suture removal and for evaluation of corneal clearing.
§ Autograft from the OD.
PTI, post transplant interval.

mens were embedded in an epoxy resin (Polybed; Polysciences, Warrington, PA), sectioned at 1 μm, and stained with azure II/methylene blue with or without basic fuchsine counterstain. To further analyze the characteristics of the corneal pathology, selected tissues were examined by electron microscopy. For this, areas were chosen from specific regions of the 1 μm-thick light microscopy sections and 60 nm sections were cut and stained with uranyl acetate-lead citrate and examined with a Zeiss EM 109 electron microscope.

After trimming, one quarter of each cornea was fixed in glutaraldehyde and formaldehyde,25 washed in cacodylate buffer, and stored in 10% buffered formalin until processed for routine histopathology. Sections of the paraffin-embedded corneas were stained with hematoxylin-eosin (H&E), periodic acid-Schiff (PAS), alcian blue (AB), and acid mucopolysaccharide (AMP) stains; the AB and AMP stains were used with or without pretreatment with testicular hyaluronidase.

Results
Clinical Evaluation of Corneal Transplants

Except for one animal (no. 3, MPS VI affected) that died of a cardiac arrest immediately after surgery, surgery and the immediate postoperative period was uneventful in the animals. All cats developed a mild to moderate vascular keratitis that was controlled with topical steroids. The steroids were used until suture removal and were discontinued shortly thereafter. Once the sutures were removed, the vessels regressed or became nonpatent and appeared as fine branching lines by retroillumination. In two animals (cats no. 4 and 6) we did not pursue vigorous treatment with topical steroids to permit the vessels to extend in one quadrant to the recipient/donor interface (Fig. 1, C, 2 and D, 2 ) and to determine whether accelerated clearing occurred in the donor buttons in areas adjacent to the neovascular infiltrates. In all transplants, the grafts remained “clear,” free of edema, and no evidence of rejection existed in any of the eyes, even those that initially showed a moderate vascular keratitis.

Both eyes of one normal cat received corneal grafts (OD, homograft; OS, autograft from fellow eye), which were followed clinically for 25 mo. Both grafts remained clear, and the donor and recipient corneal tissues were of equal thickness. Beginning 6–9 mo after surgery, fine stromal granular deposits accumulated at the host/graf t interface (Table 2). By the end of the follow-up period, these granular deposits were located in the pre-Descemet’s region (Fig. 1, A,).

The corneal disease in MPS VI-affected animals is characterized by a mild to moderate clouding that is present throughout the cornea. With biomicroscopy, the corneal stroma appears uniformly reluc tent, and storage occurs at all levels of the stroma. The diseased cornea is thinner than the normal cornea of age-matched control cats (approximately two-thirds of normal thickness5). The characteristic features of stromal storage, however, are best appreciated by retroillumination, with a biomicroscope or a hand held fundus camera. The cornea has an “orange peel” texture when viewed in this manner. Progressive disease, as occurs with aging, accentuates this texture and increases the opacity, preventing fine resolution of the iridal or fundus detail.

The transplantation experiments carried out in these cats (MPS VI recipient/normal graft or normal recipient/MPS VI graft) did not result in any clearing of the diseased cornea. These results are illustrated in Figure 1 and summarized in Table 2. When a normal
Fig. 1. Clinical photographs of experimental animals at different time points after corneal transplantation. (A) Cat no. 1 (normal with normal donor graft) at 25 mo post-transplant. Recipient and donor tissues have remained clear, but a fine granular material has accumulated at the host/graft interface. (B1) Cat no. 2 (MPS VI affected with normal donor graft) at 19 mo. The stroma in the recipient and host/graft interface have remained cloudy, with increased granular deposits within the suture tracts. Note the increased thickness of the normal cornea (B2). Air bubbles on the cornea (B1) are trapped in the tear film. (C1-C3) Cat no. 4 (normal with MPS VI donor graft) at 1.5 (C1), 10 (C2), and 14 (C3) mo. No clearing of the stromal storage occurred in the donor button, even adjacent to the vessels that course along the host/graft interface. (D1-D2) Cat no. 6 (normal with MPS VI donor graft) at 7 (D1) and 25 (D2) mo. No clearing of the stromal storage occurred in the donor button, and increased granular storage is seen within the suture tracts. (E) Cat no. 5 (normal with MPS VI donor graft) at 25 mo. There has been no clearing of stromal storage. Note that the MPS VI-affected donor button is not as thick as the normal recipient cornea.

graft was placed in an MPS VI cornea, the graft remained clear and the diseased cornea remained cloudy from stromal storage. No clearing of the recipient cornea occurred at the host/graft interface (Fig. 1, B1). As expected, the recipient corneal thickness was approximately two-thirds of the normal donor button (Fig. 1, B2). In the second experimental paradigm (normal recipient/MPS VI graft), there also was no clearing of the transplanted MPS VI-affected donor buttons, even with post surgical follow-up periods as long as 30 mo. Even in cases where mild (Fig. 1, D2) or moderate (Fig. 1, C2) vascularization developed that was limited to a focal area adjacent to the host/graft interface, focal clearing of the adjacent diseased cornea did not occur. All donor buttons remained free of edema and were approximately one-third less thick than the normal recipient cornea (Fig. 1, E). In both experimental conditions, the diseased cornea,
Table 2. Summary of clinical observations after experimental corneal transplantation

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Corneal status was evaluated on the basis of clarity and the accumulation of stromal granular inclusions: clear, no stromal storage; cloudy, corneal clouding from stromal storage; cloudy*, stromal storage limited to the pre-Desce-met's zone; gr/los, graft/host interface. Desc, relucent Descemet's membrane.

whether the recipient or the graft, showed dense accumulation of granular storage product within and adjacent to the suture tracts in the stroma, and the storage density at the graft site appeared to increase over time (Fig. 1, B, D).

Pathology—Light and Electron Microscopy

The histologic sections were analyzed for GAG storage in the cornea by evaluating in plastic sections the accumulation of cytoplasmic vacuoles indicative of substrate storage in secondary lysosomes and by examining the paraffin-embedded sections stained with AB and AMP. This analysis was made for the stroma (superficial, mid stroma, pre-Desce-met's region) in four areas: recipient tissue, donor tissue < 1000 μm from graft edge, donor tissue > 1000 μm from graft edge, and host/graft interface. The storage was compared to that in the MPS VI-affected corneas.

In the two control eyes (normal recipient/normal graft), the only abnormality found was the extracellular deposition of AMP- and AB-positive material in the pre-Desce-met's zone at the host/graft interface. This material was present around the cut end of Descemet's membrane in the area of the surgical scar. By electron microscopy, the extracellular material was found to represent homogeneous, electron dense deposits that accumulated around the keratocytes and disorganized collagen fibrils in the graft site (Fig. 2).

In plastic sections, the hallmark pathology of the MPS VI-affected cornea is the accumulation of vacuolated inclusions within secondary lysosomes (Figs. 3A, B). The keratocyte contour is irregular, resulting in a distortion of the corneal collagen fibrils that surround these cells. The keratocyte disease is not equally distributed throughout the stroma; it is most severe in the pre-Desce-met's region and least severe in the mid stroma. Storage does not occur extracellularly in the stroma, nor in the epithelial and endothelial cells. In contrast, it is more difficult to appreciate the

Fig. 2. Cat no. 1 (normal recipient/normal graft) 25 mo after transplantation. Electron micrograph of the posterior cornea adjacent to the host/graft interface shows a uniform endothelial layer (E) apposed to Descemet's membrane (D). Disorganized collagen fibrils and extracellular deposition of a homogeneous, electron dense matrix (*) are prominent. (X5980.)
extensive keratocyte storage in paraffin-embedded sections of the cornea stained with H&E or PAS. In these, the keratocytes appear slightly distended, but the inclusions are not stained. However, AB and AMP stain the accumulating storage product blue (AB = light blue; AMP = dark blue). Because most of the stored GAG is leached out during tissue processing, the blue stain usually is limited to the cytoplasmic margins of the cells and no storage is present extracellularly (Fig. 4). Hyaluronidase treatment of the sections prior to AMP staining does not affect the distribution of the stored product, although the intensity of the blue stain is slightly reduced.

Transplantation of a normal donor button into the MPS VI-affected cornea did not result in a reduction of storage in the keratocytes of the recipient cornea, adjacent to or far from the graft junction (Figs. 3A–C). Keratocytes were distended and accumulated storage product only in the affected recipient, not in the normal donor tissues (Fig. 5, compare A, 1 to B, 1). The very clear separation of keratocyte disease between the normal graft and affected recipient tissues broke down at the graft site, where diseased keratocytes were found at all levels of the stroma, and the cells showed intense AMP staining. Electron microscopic examination of the recipient cornea adjacent to the graft site showed no decrease in the severity of the keratocyte storage (Figs. 6A, B).

Similar results were found when the MPS VI-affected donor buttons were transplanted to the normal cornea. Keratocyte storage remained prominent in the cells located in the pre-Descemet's and subepithelial layers and was less distinct in the mid stroma (Fig. 7). At the three post-transplantation intervals examined (14, 21, and 30 mo), no apparent decrease in the severity of corneal disease occurred, as judged by the degree of distention and accumulation of vacuolated inclusions in keratocytes, when the cells were evaluated close to or away from the graft site (Figs. 8A–C). In addition, examination of affected donor tissues in areas where stromal vessels had infiltrated the graft showed that a reduction in keratocyte storage did not occur even in cells that were adjacent to blood vessels (Fig. 9). The AMP stains confirmed the GAG identity and indicated that storage was limited to the affected donor tissue (Figs. 10A–D).

To compare the thickness of the MPS VI-affected corneas to that of the normal, measurements were made of the PAS-stained sections with an ocular mi...
Fig. 4. Corneal button removed from MPS VI-affected cat no. 2 at 11 mo of age, before transplantation of normal donor button. Storage of GAGs within keratocytes in the anterior (A1, B1) and posterior (A2, B2) stroma results in keratocyte distention and a light (A1, B1) to dark (B2) blue staining of the cytoplasmic contents. Sections were stained without hyaluronidase pre-treatment. (A1, alcian blue; B1, AMP; ×660.)

Fig. 5. Cat no. 2 (MPS VI recipient/normal graft) 19 mo after transplantation. The acid mucopolysaccharide stain (after hyaluronidase treatment) stains blue the keratocyte storage product in the superficial and deep stromal layers of the affected recipient (A2) but not the normal graft (B2) tissues. (×660.)

crometer. These evaluations were made separately for the epithelial, stromal, and total corneal thickness in three areas: recipient, graft, and host/graft interface. The results indicate that the MPS VI cornea, whether the recipient or grafted tissue, is thinner than the normal cornea. This decrease in thickness primarily is the result of a thinner stroma (Table 3).

Discussion

Experimental corneal transplantation was performed in the feline model of MPS VI to determine whether corneal clearing occurs. In the two surgical paradigms that used affected tissues (normal recipient/MPS VI graft, three eyes; MPS VI recipient/normal graft, one eye), no improvement of the corneal disease occurred after surgery. This assessment was made clinically, by serial retroillumination examination and slit-lamp biomicroscopy over a 14-30 mo period, and histopathologically at the end of the study period.

Lack of clinical correction was based on: (1) the presence of uniformly distributed corneal stromal storage in the affected graft that did not decrease over time; and (2) the absence of clearing of the affected cornea adjacent to the host/graft junction. Subjectivity in this evaluation was minimized by using as controls other affected animals that were maintained in the colony during the period of study and by examining the unoperated eye of affected cat no. 2. These results confirm the observations we have previously published on the corneal disease in MPS VI. Lack of histopathologic correction was based on the presence of stored, undegraded substrate in keratocytes, which appeared as vacuolated inclusions in aldehyde/osmium-fixed, plastic-embedded tissues, or as blue reaction product in AB- or AMP-stained sections. This storage was limited to the keratocytes of MPS VI-affected tissues, whether graft or recipient, and failed to show any difference in the severity of the storage in samples analyzed close to or far from the host/graft interface. Although morphometric evaluation was not performed to assess the effects of transplantation on the keratocyte disease—e.g., quantitation of the number of secondary lysosomal inclusions per cell or

Fig. 6. Cat no. 2 (MPS VI recipient/normal graft) 19 mo after transplantation. Electron micrographs of the superficial (A) and deep (B) corneal layers taken less than 1000 μm from the graft site. Keratocytes (arrows) are distended by the intracytoplasmic accumulation of inclusions that are predominantly vacuolated. The severity of the disease is no different than in more peripheral corneal regions. Epi, epithelium. *, extracellular accumulation of a homogeneous, electron dense matrix. D, Descemet's membrane. (×5250.)
Fig. 7. Cat no. 6 (normal recipient/MPS VI graft) 30 mo after transplantation. Electron micrographs of the superficial (A) and deep (B) corneal layers of the MPS VI-affected graft. Keratocytes (arrows) located less than 1000 μm from the host/graft interface show accumulation of intracytoplasmic inclusions. Ep, epithelium. (A, ×7600; B, ×5075).

Fig. 8. Cat no. 6 (normal recipient/MPS VI graft) 30 mo after transplantation. Section of the superficial (A₁, B₁, C₁) and deep (A₂, B₂, C₂) corneal layers of the recipient (A₁, B₁, C₁) and donor (A₂, B₂, C₂) tissues adjacent to the graft site, or the donor tissue >1000 μm from the graft site (C₁, C₂). Accumulation of vacuolated inclusions in keratocytes (arrows) is limited to the MPS VI-affected donor tissues (B₁, B₂, C₁) and is most prominent in the superficial and deep layers of the stroma. There is no difference in the severity of storage in cells located adjacent to or away from the surgical interface. *, reduplication of Descemet's membrane. (×475.)
The mechanism responsible for the corneal thinning is unknown, but may result from impaired corneal collagen synthesis, fibril formation, or altered extracellular matrix biosynthesis. These may result directly from the enzyme deficiency or indirectly from the storage of undegraded substrate within the keratocytes.

Prior studies in MPS affected animals and human patients have reported that corneal clearing occurs in some patients after partial restoration of deficient enzyme activity by bone marrow transplantation. One report reviewed results in man and indicated that corneal clearing occurred in MPS I, II, IIIb, IV, and VI, while other reports indicated that, at least in MPS VI, no significant corneal clearing occurred after bone marrow transplantation. In contrast, two MPS VI patients who underwent successful corneal transplantation to correct the corneal clouding showed clearing of the recipient cornea adjacent to the graft site. These differences may be methodological, in the criteria for evaluation or in the degree of restoration of deficient enzyme activity, or may underscore the basic biochemical differences that exist within and between the distinct MPS disorders. A similar situation exists in animals where the early reports that cite corneal clearing in MPS VI cats have not been confirmed on follow-up studies.

The mechanism of corneal clearing in MPS disorders after bone marrow transplantation is postulated to involve enzyme replacement to the deficient keratocytes. The normal enzyme is released from the hematopoietically derived donor cells and enters the keratocytes by a receptor-mediated internalization process with subsequent targeting to the lysosomal compartment. There, the active enzyme is able to degrade stored substrate and prevent the subsequent GAG accumulation in the secondary lysosomes.

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Epi, epithelial thickness, in microns. Str, stromal thickness, in microns. Total, total corneal thickness, in microns.
cause the cornea is avascular, the enzyme must diffuse through to the stroma from the limbal vessels, the pre-corneal tear film, aqueous, or all three sites. At least for MPS I, the tears are a ready source of enzyme that can be used for diagnostic purposes or evaluation of bone marrow transplantation.27

We used reciprocal corneal transplantations to evaluate the usefulness of corneal clearing as an index of successful bone marrow transplantation and disease correction. We reasoned that transplanting an MPS VI-affected corneal button into a normal eye, where all cells and tissue fluids have normal enzyme activity, would provide the most optimal medium to facilitate the correction of the GAG catabolic block and result in corneal clearing. The eye of a normal animal, compared to that of an affected animal treated by bone marrow transplantation, would have the highest level of enzyme in the corneal tissues, the aqueous, and tears. Such high tissue enzyme activity levels are unlikely after bone marrow transplantation in a deficient patient, because bone marrow transplantation primarily restores enzyme levels in circulating white blood cells, other bone marrow-derived cells, and serum. However, corneal clearing did not occur in MPS VI-affected tissues, whether graft or recipient. The lack of corneal clearing, primarily by diffusion from the vascular compartment, was further supported by the observations made in two animals that

Fig. 10. Cat no. 5 (normal recipient/MPS VI graft) 21 mo after transplantation. Recipient stroma shows absence of keratocyte storage away from the graft site (A, B), but storage of AMP-positive material in the grafted tissue (C, D). Keratocyte storage is most distinct in the AMP-stained section (C). (A, C, AMP after hyaluronidase pretreatment; B, D, hematoxylin-eosin; x363.)
showed no clearing even in areas of the graft that were adjacent to corneal vessels that extended to the host/grant junction. These are areas that would be expected to have high levels of enzyme activity.

Failure of corneal clearing in these experimental transplantations could result from one or a combination of different mechanisms: low levels of enzyme in the aqueous or tears, insufficient enzyme levels in extracellular compartments, failure of diffusion of enzyme from the normal to the affected cornea, or failure of the MPS VI-affected keratocytes to internalize the secreted extracellular enzyme. Studies have not examined these questions in detail, but these issues are readily addressed using the animal models described in the present experimental studies.

**Key words:** animal model, arylsulfatase B deficiency, cat, corneal transplantation, Maroteaux-Lamy syndrome, mucopolysaccharidosis VI.

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**References**