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Use of penetrating keratoplasty in equine ophthalmology

Extraction of the PhD dissertation

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Introduction

Ulcerative and non-ulcerative keratitis are common eye disease in horses that threaten the visual ability. Infectious agents can invade the corneal stroma in the case of inflammatory keratopathies and induce infiltration of neutrophil granulocytes, slow epithelisation, intensive neovascularisation, rearrangement of collagen fibrils and scarring. In case of fungal infection the inflammation advances toward the Descemet-membrane (DM), and the structure of the stroma is destroyed due to the microbial activity. Matrix metalloproteinases (MMP-2, MMP-9), elastase, protease enzymes damage the cornea causing melting ulcer or abscess, furthermore induce severe secondary iridocyclitis.

Keratoplasties in the horse have curative purposes to eliminate inflammatory processes or to maintain corneal integrity (tectonic reasons) but not to improve vision. Therefore keratoplasty is performed when the conservative treatment of keratitis with drugs had failed and in order to eliminate the simultaneous iridocyclitis or to restore ocular surface. The conservative treatment is considered unsuccessful when there is no improvement by 48 hours after its start regarding the size of the corneal lesion or the intensity of vascularisation and anterior uveitis. The main indications of penetrating keratoplasty (PK) in the horse are: excision of the lesion in the deep stroma (stromal abscess – SA; endotheliitis; neoplasia), and the maintenance of the corneal integrity, replacement of the damaged part of the cornea in case of a deep melting ulcer, descemetocele or perforating wound.

PK is a complete microsurgical transplantation of the entire corneal thickness, including excision and replacement of its all layers. Penetrating keratoplasty can be performed successfully under general anaesthesia that provides about 80% of visual ability in most of the cases besides graft scarring.

Purposes

1. To examine how the donor corneal graft (allogenic transplantation) can be healed in the healthy recipient equine cornea after penetrating keratoplasty.
2. To explore the differences between the final results of healing after usage freshly harvested, preserved and frozen corneal grafts.
3. To examine the feasibility of usage of preserved corneal allografts for PK in horses.

4. To measure tear immunoglobulin levels before PK surgery and in the early postoperative period and to follow their changes in time as well as to compare the values from the operated and non-operated eyes.
5. To discover the possible correlations between postoperative uveitis, measured immunoglobulin levels, degree of immunorejection, and the final clinical result.
6. To summarise the clinical experiences regarding the difficulties of the surgical technique and its adaptation to horses and the postoperative management.

Experimental penetrating keratoplasty and graft immunorejection

Materials and method

Eight horses owned by the Large Animal Clinic, Veterinary Faculty were included in the study (Case 1-8.). Every horse had complete deworming and vaccination protocol. The horses were Hungarian half-bred, aged between 3-7 years. In two cases (Case 4, 7.) the right eye, and in six cases the left eye was operated. Five preserved (Case 1-5.), two fresh (Case 6, 7.) and one frozen (Case 8.) corneal allograft were utilised. The horses underwent complete ophthalmologic examination including slit-lamp biomicroscopy, direct ophthalmoscopy and tonometry, and both eyes proved to be healthy.

Preparation of the donor cornea

Donor horses were Hungarian half-breeds, aged 10-13 years, and euthanized for unrelated reasons at the clinic. These horses were free of contagious diseases or neoplasia and should have healthy eyes. The following three methods were used to store corneal grafts:

1. Fresh whole eye globes excised after euthanasia (within 6-24 hours after death). The enucleated eye globe was kept in a refrigerator at +4°C for maximum 2 days in a sterile moist-chamber (case 6-7).

2. Thirteen corneas were preserved according to the Budapest Cornea Bank protocol. The donor corneas were harvested and excised sterile from healthy eyes of horses aged from 6 months to 13 years. The average time between death and excision was 7 hours (0.5 – 14.5 hours). After euthanasia the ocular surface was rinsed with 0.5% Betadine solution and 0.3% gentamycin solution and the entire cornea with a scleral margin was excised from the eyeglobe with a scalpel blade (case 1-5). After excision the endothelial cell density was checked with non-contact specular microscope and found 2400-3000/mm². The corneas

were preserved in tissue culture media (Medium I and II.) according to the Human Cornea Bank protocol (Budapest, since 1993). The cornea can be preserved in Medium I. for several weeks, but before utilisation they should be put into Medium II. at least for 3-7 days. This latter media contains 5% dextran and has higher pH and osmolarity to keep cornea dehydrated. The whole preservation period can last for maximum 30 days. The endothelial cell loss and destruction should be less than 10 % during preservation. Samples for bacteriology and mycology were submitted altogether on two occasions during preservation. For the first time on the 5th day from Medium I., and for the second time at putting cornea to Medium II. In total 13 corneas were preserved, from which that were utilised that had negative microbiological culture and the liquids remained completely clear and transparent throughout the preservation period. The cause of euthanasia, the time of corneal excision, the gender, breed, age of the donor horse and the culture results of the medias, the phases and duration of the preservation time were recorded in the 1-5. cases.

3. In case of frozen preservation the eye globes were excised from cadaver horses within 6 hours after death and rinsed with 0.3% gentamycin solution, then kept in a deep freezer at minus 20°C. They were allowed to thaw for couples of hours before usage at room temperature (case 8).

Determination of immunoglobulins from tear

100 µl of tear was collected with glass capillary tube from the medial meniscus of both eyes at each sample while avoiding reflex tearing but without topical anaesthesia, between the daily treatments. The samples were taken before the surgery (day 0), and then on the 3rd, 6th, 9th, 12th and 21st days after surgery, respectively. The tear was put from the glass capillary tube into an Eppendorf tube, then immediately frozen and kept at minus 80 °C until analization of all samples. IgA, IgG and IgM levels were measured from the tear samples with direct ELISA according to standard protocol. We examined whether the immunoglobulin levels of tear differ in the operated eye from the control fellow eye and their changes in time regarding each immunoglobulin type. Tears from clinically inflammed eyes were compered to less inflammed ones.

Results

Clinical results

Five donor corneas preserved in tissue culture media (case 1-5), 2 fresh (case 6. and 7), and 1 frozen (case 8) were utilized. Corneas were preserved for 11 days in cases 1 and 2; for 10 days in cases 3. and 4; and for 16 days in case 5. Blepharospasm and corneal edema around the graft were noticed on the first postoperative days in each operated eye. Later the

transparency of the graft was estimated by taking photos and written findings and it was described as transparent or edematous or fibrotic.

On the third postoperative day mild, moderate or severe blepharospasm was detected and mucoid discharge was noticed in cases: 1, 2, 5, 6, 8. The corneal grafts were more edematous and bulged out from corneal surface, and the cornea adjacent to the graft was also edematous. On the 3rd-4th postoperative days the topical tobramycin was switched to chloramphenicol in cases: 1, 2, 5, and to ciprofloxacin in case 8. This was indicated because of the mucopurulent discharge from the eyes and the results of the microbial culture and antibiotic susceptibility test (case 8).

At the beginning the graft site was pink because of the neovascularisation and the cellular infiltration, which are the concomitant signs of the healing process. Superficial vessels coming from the limbus toward the graft site were always visible at each case from about the 4th-5th postoperative days. The graft was significantly swollen in the first 2 weeks postoperatively, and as a subepithelial bulla was outbulging from the cornea, oftentimes 1 mm from the surface. The clinical signs of iridocyclitis, such as miosis, aqueous flare, deep stromal vascularisation and edema were always remarked in all horses, but in different extent (significant anterior uveitis: case 1, 5, 8) in the first 21 days. By the 2nd-3rd postoperative week there were no fluorescein stain uptakes and the epithelialisation has been completed. Marked corneal fibrosis and pigmentation of the graft and adjacent cornea were seen at around the 42nd day. The grafts remained opaque and vascularised, but by this time the edema and bulla formation had ceased. The grade of pigmentation usually correlated with the severity of inflammation (eg. case 2: slight inflammation and no pigmentation; case 8: intensive inflammation and increased pigmentation). Certain degree of graft immunorejection was always noticed, eventually none of the horses had completely transparent graft. The average surgical time was 94 minutes. The postoperative complications and sequelae were as follows: slight blepharitis due to the SPL built into the dorsal conjunctival fornix in case 1, 3, 8.; infectious keratitis caused by *Pseudomonas aeruginosa* developed in case 8 on the 7th postoperative day, which was diagnosed based on the results of bacterial culture. This time the topical chloramphenicol was switched to ciprofloxacin according to the susceptibility test, and the severe clinical signs related to the keratitis has been ceased by the 21st day. The other operated corneas healed without infection. Anterior synechia was detected in case 7 at around the 42nd day. In 2 cases (case 3, 5) mild corneal surface irregularity and aberrant curvature of the cornea were experienced, so significant astigmatism developed in these 2 cases. Every eye was followed at least for 180 days after surgery, the mean follow-up time was 286 days (180-425 day). In each case the visual ability remained 100 % according to the intact direct PLR, dazzle and menace response at the last recheck.

Statistical analysis

The measured immunoglobulin levels of the tear samples from operated and non-operated eyes were compared with t-test. Referring to the measured IgA levels, the differences had normal distribution and the values of the non-operated eyes were significantly less ($p=0.001$). There was no significant difference in time relation ($p=0.812$).

Referring the measured IgG values, the levels had normal distribution, and the difference was not significant ($p=0.101$). There was no significant difference in time relation ($p=0.221$).

Referring the measured IgM values, the differences had normal distribution, and the values of the non-operated eyes were significantly less ($p=0.006$). There was no significant difference in time relation ($p=0.217$).

Linear regression analysis was used when immunoglobulin levels of the operated and non-operated eyes were compared in time relation. None of the immunoglobulins had significant change in time relation in the operated eyes: IgA analysis: $p=0.109$, IgG analysis: $p=0.1929$, IgM analysis: $p=0.7823$.

Using linear regression analysis, there were no significant changes in time relation in the cases of non-operated eyes. IgA analysis: $p=0.513$, IgG analysis $p=0.9566$, IgM analysis $p=0.1873$.

The IgG levels of the tear samples in the operated and non-operated eyes were lower, when compared to the IgA and IgM levels.

Discussion

For PK surgeries we used not only fresh corneas that were harvested within 24 hours after euthanasia and utilised within 48 hours, but frozen and in tissue culture media preserved ones, too. Adequately incorporated corneal grafts were obtained, however all remained opaque. In cases 1-5. preserved corneas, in cases 6, 7. fresh corneas, and in case 8. a frozen cornea was used as donor tissue. In everyday practice the most easily approachable and cheapest preservation technique is freezing. This method allows availability of frozen donor corneas in a clinic at any time. From our cases the case 7. can be determined as the most successful clinical outcome, where the donor corneal graft originated from a recently died horse's eye, harvested within 1 hour after death and was sutured with USP 8-0 nylon suture material. Based on the postoperative clinical findings, the differently preserved corneal grafts incorporated in the same manner and seemed to be the same by inspection after the first 1-2 weeks.

Every PK surgery is considered successful, because the donor grafts incorporated properly into the recipients tissue. In the case 6. we used simple interrupted suture combined

with simple continuous suture pattern that seemed to be safe enough to hold the graft in place and less irritable due to fewer knots, but using simple interrupted suture is the safest technique to perform PK surgery in horses. Especially when the graft is sutured in an infected recipient cornea. One of the most common postoperative complications of the PK surgeries in infected eyes, is the suture dehiscence. The non-absorbable suture material (USP 8-0 nylon, case 6-8) was not intentionally removed, because it didn't cause any kind of discomfort or interfere vision. Monophyl suture material causes less irritation than polyphyl absorbable one, and this rather can be reduced, if the knots are turned in and hidden in the corneal tissue.

Based on our experiences the deficiencies of the surgical technique (eg. not adequate graft manipulation, too superficial or perforating stitches) and the postoperative management are determining factors of successful outcome. With more complications the surgical site can heal with more prominent and greater scar. Administration of topical and systemic antibiotics, antimycotics, topical mydriatics-cycloplegics and NSAID drugs for several weeks is essential to provide proper healing. After PK surgery the patient's eye should be closely monitored in order to reveal complications, like secondary uveitis, as early as possible. The secondary anterior uveitis as a consequence of keratitis in horses, is the result of a sensory-nerve irritation related to the trigeminal nerve, which is called axon-reflex-activation and induce secondary iridocyclitis. It is important to prevent periocular and SPL damages from the face rub using protective hood or mesh in the postoperative period.

The clinical manifestation of immunorejection is the corneal graft opacity and infiltration. This study confirms the results of previous studies, so the vascularisation, cellular infiltration, edema, and late fibrosis with certain pigmentation of the corneal graft in horses are concomitant features of graft incorporation. The corneas of the study horses were completely healthy, transparent and avascular before PK surgery, so the chance for immunorejection was minimal. The used fresh or preserved corneal grafts contained live, intact endothel. Nevertheless none of the grafts remained transparent postoperatively. After our surgeries it is evident, that the corneal vascularisation after PK surgery is important and necessary to the incorporation and was observed in each case, that process contributed both to the healing and immunological exposure, secondary immunorejection. Our results to retain corneal integrity and preserve vision were successful.

Immunoresponse

Components of the complement system, regulatory proteins, IgG and IgM molecules can be demonstrated from healthy human tear. The elevation of IgA- and IgM-levels considered to be very specific predilecting factors at graft rejection, but other immunological reactions (inflammation due to infection) also can contribute to the increased secretion of the local

immunoglobulines. The permeability of the conjunctival vessels increased due to the significant inflammation in our cases. The IgM level also increased subsequently even though it has a great molecular weight.

It has a major role in the outcome of a PK surgery in humans, if the recipient cornea has already been vascularised before surgery. In these cases there is a great chance, that the graft won't be transparent any more. Presumably the local cellular and humoral immunity are responsible for the acute and chronic rejections after PK. The increase of the tear IgA- and IgM-levels predict the clinical signs of the rejection in humans and indicates the optimal time to initiate immunosuppressive treatment (local and systemic corticosteroid drugs), which is about at 12th postoperative day. After detection of increased tear immunoglobulin level, the immunosuppressant therapy has been started in right time, the reaction won't proceed. In horses, when PK is indicated, the cornea has already been vascularised, and the vascularisation is unavoidable after surgery too, which determines the local cellular and humoral immunresponse. In our study the measured IgG-levels were lower than IgA- and IgM-levels, in contrast with levels measured in humans, where IgM had a low basic level. IgG had a low basic level in each tear sample of horses, the IgA- and IgM-levels had great variability in time. Regarding the IgA- and IgM-levels, the values of the non-operated eyes were fewer compared to operated ones. Regarding the IgG levels, there was no significant difference between the values of the operated and non-operated eyes. There was no significant difference in the levels of different immunoglobulin levels of the operated or non-operated eyes in time relation. The Ig-levels rather had a typical individual pattern in both eyes in a certain horse. The IgA- and IgM-levels were similar in one eye. Some IgA peaks were detectable at around the 6th day, which is the result of a local antigen stimulus. This IgA-level may represent local immunactivity the most in horses that seems to be earlier, than in humans, which is at around 12th day. IgM- and IgA-levels of the tear could raise due to the local immunstimulation and inflammation. On the other hand, a surgical procedure performed on one eye may play a role in the influence of the immunresponse of both eyes. The MMP-2 levels of healthy contralateral eyes of horses had increased values in a study where normal control eyes were compared to the contralateral eye that had had an ulcer at the same time. This may be the consequence of a central or systemic sympathetic response on the eye, when an inflammation developed on a certain mucus membrane may induce a leukocyte migration on a thus far intact mucus membrane and may initiate a response in the conjunctiva associated lymphoid tissue (CALT) of the other eye, that finally activate the resident lymphocytes there. There was no significant difference between horses, when very inflamed and slightly inflamed eyes were compared concerning the immunoglobulin levels. This can be, because the measuring technique was not enough sensitive, so other

inflammatory proteins might be remained in the tear samples, while the actual immunoglobulin-levels were just determined on a rough estimate.

Use of penetrating keratoplasty for the treatment of corneal abscess and in other clinical cases

In the second part of our work three clinical cases were introduced and described in which the reason for PK was thorough thickness corneal abscess. In the case descriptions the history, clinical signs, allograft transplantation, and the postoperative outcome were specified. The abscess invading whole corneal thickness was excised in all three cases. The abscess was excised in a round shape with a corneal trephine 1 mm larger than the lesion, and the graft was sutured into the recipient cornea. A fibrotic graft remained in every case, but all globes had light perception and in 2 cases remained visual. PK is a feasible and effective microsurgical procedure to treat stromal abscess in the horse, since the topical treatment of these abscesses are ineffective. The postoperative result is also favourable, with an early surgery the vision can be preserved and good cosmetic result can be obtained. Not any publication has been come out so far in the Eurapen literature that would have described PK to treat corneal abscess in the horse.

In the third part of our work other six clinical cases were described, where all indications for PK occured. With the help of PK – even with conjunctival flap combination – successful light perception, in some cases good vision remined even in coreal perforation. All grafts incorporated well in the recipient, but a remarkable fibrotic opaque scar remained at the graft site. Every horse underwent PK surgery could come back to previous work, their eyes were comfortable, without tearing nor blepharospasm at the last recheck.

Conclusions

Penetrating keratoplasties performed on 8 healthy and 9 clinical patient's eye prove that the opacity and immunrejection of the graft began typically on the 5–7th day after surgery, and allways occured, we were not able to ward it off. Even in cases of healthy eyes, the grafts became opaque and pigmented as a result of immunrejection. The prominent cause of graft immunrejection in the horse is still unknown, but it is certain, that considerable inflammatory and immunological processes are going on at the time of corneal discontinuity, as IgA- and IgM-levels of tear raised after surgery. Because of this, it is very unlikely, that completely

transparent grafts could remain in horses that are expected in humans after PK. A small corneal scar can be considered good result in the horse. However in humans because of the scar another surgery might be needed if the graft is put to an inflamed cornea, when the cause of reoperation is not tectonic, like in horses, and the position of the scar is central. The term “corneal graft” or „corneal patch” can be better in horses than corneal transplantation to designate this type of surgery, because in these situations a relatively small, usually frozen piece of corneal tissue without immunological matching is used to replace the excised lesion. The most practical method is to use frozen grafts with damaged endothelial cells, when these grafts can be stored at minus 20 C° for 6 months and then utilised. From our observations the not completely thawed donor graft can be handled and sutured easier. The most appropriate graft is from a foal eye, still containing intact, living endothelial cells, but this also will be completely opaque after surgery. Although getting fresh grafts is incidental, preservation method in tissue culture media is very expensive and the usage time is limited to couples of weeks. There is no real cosmetic difference after usage of differently harvested grafts. Use of automatic microkeratom, new antimicrobial and antimycotic and immunosuppressant drugs may improve the PK outcomes in horses. Improvement of all these things may result in a more transparent graft in horses underwent PK surgery in the future. Experimental PK surgeries in dogs support our experiences, that the success rate of the surgery is mostly dermined by the suture of the cornea, the surgeon’s skill, and the endothelial cell density. The spread and further development of different keratoplasties in equine ophthalmology will help to reduce the sequelae of severe corneal problems and promise more favourable prognosis.

New scientific results

1. We described the usage of donor corneal grafts preserved in tissue-culture media for PK surgery of horses for the first time.
2. Even fresh or preserved corneal grafts remained opaque when used for healthy, transparent, avascular recipient corneas in the horse.
3. Fresh, preserved or frozen donor grafts are incorporated in very similar manner and can be utilised equally for PK surgery in horses with the same cosmetic outcome and success.
4. The secondary anterior uveitis also develops after PK surgery of healthy corneas.

5. We determined the immunoglobulin levels of equine tear for the first time. IgG-level remained at a basic low level, but after PK surgery the level of IgA and IgM showed raise between the 3-10th postoperative day in the operated eyes.

Publications

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