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Evaluation of Active and Passive Transport Processes in Corneas Extracted from Preserved Rabbit Eyes

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Abstract

In vitro transcorneal permeability studies are an important screening tool in drug development. The objective of this research is to examine the feasibility of using corneas isolated from preserved rabbit eyes as a model for permeability evaluation. Eyes from male New Zealand White rabbits were used immediately or were stored overnight in PBS or HBSS over wet ice. Integrity of isolated corneas was evaluated by measuring the TEER and by determining the permeability of paracellular and transcellular markers. Active transport was assessed by measuring transcorneal permeability of selected amino acids. Esterase activity was estimated using p-nitrophenyl assay. In all cases, corneas from freshly enucleated eyes were compared to those isolated from the day-old preserved eyes. Transcellular and paracellular passive diffusion was not affected by the storage medium and observed to be similar in the fresh and preserved eye models. However, amino acid transporters demonstrated lower functional activity in corneas excised from eyes preserved in PBS. Moreover, preserved eyes displayed almost 1.5-fold lower esterase activity in the corneal tissue. Thus, corneas isolated from day-old eyes, preserved in HBSS, closely mimics freshly excised rabbit corneas in terms of both active and passive transport characteristics but possesses slightly reduced enzymatic activity.

Keywords

in vitro models; paracellular transport; permeability; tight junction; transcellular transport; transporters

INTRODUCTION

Transcorneal absorptive pathway is the most important route of absorption for drugs applied topically to the eye[1]. A major challenge for pharmaceutical scientists attempting drug delivery to the anterior segment of the eye, however, is overcoming the epithelial tight junction barriers[2] and also tailoring the pharmaceutical properties of the compound to meet the diffusional restrictions imposed by the unique corneal structure comprising of both hydrophilic and lipophilic layers. To exert therapeutic activity, a topically administered agent must demonstrate sufficient corneal permeability so as to generate adequate drug

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concentrations at the site of action in the anterior ocular chamber. Thus, *in vitro* transcorneal permeability studies play a critical role in the screening and selection of drug molecules and formulation components during the design phase.

Compared to *in vivo* experiments, *in vitro* experiments avoid and isolate confounding physiological mechanisms from transmembrane diffusion; are less time consuming; amenable to high throughput screening; and are thus preferred. Currently, the most favored model for *in vitro* corneal permeability determinations are freshly excised animal corneas[3].

Rabbit eyes are physiologically very similar to the human eyes and have been extensively used to study corneal permeation of therapeutic moieties and to optimize topical formulations before preclinical and clinical testing. Unfortunately, these experiments require sacrificing of animals procured specifically for the purpose of *in vitro* testing and also incur high costs associated with the caring for laboratory animals[4]. In some cases investigators use corneas isolated from rabbits used in other protocols. This practice does reduce the number of animals required but leaves the investigators unsure about the number and time of availability of the tissues and suffers from a lack of control on parameters such as age and sex. Moreover, the experiments carried out in the original protocol may affect ocular permeability characteristics. It would be a significant step towards reducing the number of animals required for research purpose if the pressing need to specifically sacrifice rabbits for the purpose of carrying out *in vitro* transcorneal experiments could be eliminated.

Cell cultures, as *in vitro* models, have not met with much success with respect to projection of corneal permeability rates. This is primarily because of the lack of tight junction expression, variability in the expression and polarization of transporter proteins[5] and difficulty in accurately mimicking the multi-layered and multi-component corneal structure in the currently available cell culture models. The cell lines also exhibit time dependent TEER values, have a high cost associated with producing and maintaining the cultures and are susceptible to microbial contaminations[6]. Becker *et al*. have compared different epithelial cell culture models available as well as corneal constructs for *in-vitro* drug permeation[7]. The authors compared Statens Serum Institute Rabbit corneal cells (SIRC), transformed human corneal epithelial cells (HCE-T) cell lines as well as commercially available SkinEthic reconstituted human corneal epithelium (HCE-S) and Clonetrics cultured human corneal epithelium (HCE-C). The results indicated that SIRC, HCE-S and HCE-T could not differentiate between the permeabilities of molecules with different physicochemical properties. Only HCE-C remained a viable option but has to be used within 24 hours of receipt from the manufacturer. Since ^{14}[C] mannitol permeability across HCE-C was not reported a direct comparison cannot be made between the corneal tissue and the HCE-C cell line in terms of the paracellular diffusional barrier properties. Moreover, the authors did not evaluate the cell culture models with respect to molecular expression and functional activity of carrier-mediated systems, which plays a critical role in transcorneal diffusion.

Alternatively, eyes obtained from local abattoirs have been evaluated for studying transcorneal permeability[8-10]. Various means to transport the excised eyes from the slaughterhouse to the laboratory have been reported. Eyes have been transported directly on

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ice or have been placed in solutions and transported over ice[8-10]. In all studies, the eyes were used within a couple of hours post isolation and knowledge about the effect of prolonged storage is currently lacking. Considering that the abattoirs would sacrifice the animals only once or twice a week, an option for preserving the procured eyes overnight in specified solutions, would significantly help in the research process as well as bring down the need for sacrificing laboratory animals. Storage may, however, lead to alterations in the corneal epithelial structure and protein expression which would impact permeability of drug molecules. To date, the effect of the storage conditions and storage solutions on corneal integrity and permeability characteristics has not been fully investigated.

Literature indicates that eyes stored in phosphate buffered saline (PBS) and hanks balanced salt solution (HBSS) have been successfully used for the preparation of primary cell cultures[11-13], indicating that corneal cells remain viable in these medium for the duration tested. However, for these studies, maintenance of the integrity of the corneal epithelial barriers was not important and was thus not tested. Corneas stored in unfavorable media would swell, display decreased integrity due to the loss of tight junction proteins and may also demonstrate diminished functional activity of transporters expressed on the cornea. As a result studies with these tissues may predict erroneous permeability values for the test compounds.

Incidentally, a lot of research has been focused on developing media for the storage and transportation of human corneas. Preserved corneas have been evaluated with respect to biological and histological characteristics[14-17]. Although corneas are shipped for transplantation in human subjects, the storage / transport solutions available are very expensive and the sheer volume required for shipping whole animal globes makes these media even less attractive for *in vitro* corneal permeability studies in the drug development process. Moreover, surprisingly, the storage solutions currently used have never been evaluated with respect to the preservation of the corneal epithelial barrier and transporter characteristics.

In this study, we have evaluated PBS and HBSS, two commonly used cell culture media, as storage solutions for whole rabbit eye globes. Mannitol and diazepam have been routinely used as markers for passive paracellular and transcellular pathways and were thus selected for this study. Additionally, corneal permeability of the hydrophilic nucleoside antiviral agent, acyclovir, known to diffuse across the cornea by passive diffusion mechanisms[18], was also studied. Quinidine, a lipophilic compound traversing biological membranes by the transcellular route using a combination of passive and active diffusional processes[19], was also included as a marker compound. Moreover, the functional activity of the three amino acid transporters previously reported on the corneal epithelium: the $B^{0,+}$, ASCT1 and L-type amino acid transporters were also tested[20-22]. In addition to passive and active transport processes, activity of esterase enzymes was also compared since these enzymes regulate bioreversion of ester prodrugs in the corneal tissue and, thus, can impact net corneal permeability. It was assumed that since the eyes would generally be preserved over wet ice, the metabolic rate would be minimal. Thus, addition of nutrients, e.g. essential amino acids, would not be necessary. Also, since whole animal globes, rather than isolated corneas, would generally be preserved, no osmotic agent such as dextran was added to the medium.

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EXPERIMENTAL SECTION

Materials

Acyclovir was obtained from Hawkins Inc. (Minneapolis, USA) and quinidine from Sigma Chemical Co. (St. Louis, USA). Radiolabelled amino acids were obtained from Moravek Biochemicals, Inc. (Califonia, USA) and [^{14}C]mannitol and [^{3}H]diazepam were from Perkin Elmer Life and Analytical Sciences (Boston, USA) respectively. All other solvents and chemicals were obtained from Fisher Scientific (Fair Lawn, NJ), and used as such.

Animals

Male, albino, New Zealand rabbits weighing between 2 to 2.5 kg were procured from Myrtles' Rabbitry (Thompson Station, TN). Animal experiments conformed to the tenets of Association for Research in Vision and Ophthalmology (ARVO) statement on the Use of Animals in Ophthalmic and Vision Research.

Methods

Corneal Permeation Studies—Animals were anesthetized with ketazime/xylazine given intramuscularly and euthanized by an excess of pentobarbital injected through the marginal ear vein. Some of the globes so obtained were either stored in PBS or HBSS, over wet ice, or were taken for immediate isolation of the corneas and further experimentation. Corneas from the preserved globes were isolated 24 hours after storage initiation. Corneas were excised, following previously published protocols [23], with approximately 1 mm scleral portions remaining for ease of mounting. The corneas were mounted between standard, 9 mm, side-by-side diffusion cells (PermeGear Inc., Bethlehem, PA) with the epithelial layer facing the donor side. Temperature was maintained at 34°C during the transport studies with the help of a circulating water bath. Dulbeccos phosphate buffered saline (DPBS) was used as the transport medium. Volume of the receiver solution (3.2 mL DPBS) was slightly higher than that of the donor solution (3 mL drug solution) to maintain the natural curvature of the cornea. Contents of both chambers were stirred continuously. Aliquots, 200 μL, were withdrawn at appropriate time intervals and immediately replaced with an equal volume of DPBS and stored at -80° C until further analysis. Unlabeled samples were analyzed using an HPLC system.

To the radioactive aliquots five milliliters of scintillation cocktail (Scintisafe Econo 2, Fisher Scientific, USA) was added and the radioactivity was measured using a Liquid Scintillation Analyzer (Perkin Elmer Life and Analytical Sciences, Model TriCarb 2900TR, CT, USA).

Light Microscopy

Corneas were stained with hematoxylin-eosin using previously published procedures[7]. Briefly the corneas were dehydrated in increasing ethanol concentration, embedded in paraffin wax and then cut into 4μM sections. These sections were stained with hematoxylin and eosin solutions and used for light microscopy.

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Trans-epithelial Electrical Resistance (TEER)

Ag/AgCl electrodes 2 mm in diameter were shaped in the form of circular rings and placed approximately 2 mm from the cornea in both the donor and the receiver compartments and the chambers were filled with DPBS solution. The electrical resistance across the corneas was measured, every hour for a period of three hours, using an experimental setup consisting of a waveform generator and digital multimeter (Agilent Technologies, Santa Clara, CA).

Paracellular and Transcellular Permeability

Tight-junction characteristics was compared using acyclovir (1mM) and [^{14}C]mannitol (0.5 μCi/ml, specific radioactivity 55 mCi/mM) in DPBS, as paracellular diffusion markers. The transcellular permeability markers quinidine (0.5μM), and [^{3}H]diazepam (0.5 μCi/ml, specific radioactivity 70 mCi/mM) in DPBS, were used to compare the properties of the lipoidal cell membrane. The studies compared fresh versus preserved corneas.

Transport Activity of Amino Acid Transporters

To compare functional activity of corneal amino acid transporters, transcorneal permeability of [^{14}C]L-Arginine (specific activity 57 mCi/mM), [^{14}C]L-Phenylalanine (specific activity 391 mCi/mM) and [^{14}C]L-Alanine (specific activity 162 mCi/mM) was determined in both freshly excised and preserved rabbit corneas. L-Arginine and L-phenylalanine are known to be transported solely by the $ATB^{0,+}$ and LAT1[20,21] transporters, respectively. Although multiple systems are involved in the transport of L-alanine, it permeates across the rabbit cornea primarily through the ASCT1[22] transporter. Procedures as described under corneal permeation studies were followed. The donor solutions contained 0.5 μCi/mL of the radioactive agents.

Choline chloride and K_2HPO_4 were used in equimolar quantities to replace NaCl and Na_2HPO_4, respectively, in the transport medium to study sodium dependency of the transport process. 2-Amino-2-norbornanecarboxylic acid (BCH) was used as a specific L-amino acid transporter inhibitor.

Enzymatic (esterase) Activity

A method described by Armstrong *et al*. was modified to determine the esterase activity in the ocular tissues obtained from fresh and preserved rabbit eyes[24]. Eyes were carefully dissected and the isolated tissues were stored at -80°C. Aqueous humor and vitreous humor were used after centrifugation at 13,000 rpm at 4°C for 5 minutes. Cornea, iris-cilliary body and retina-choroid were homogenized over an ice bath by using methods described elsewhere[25]. The protein content of the tissue homogenates was standardized to 1 mg/mL using the method of Bradford[26]. Total esterase activity was determined spectrophotometrically using p-nitrophenyl acetate (3 mM) as a substrate. In 3 mL acetone, 54.3 mg p-nitrophenylacetate was dissolved and the volume was made up to 100 mL using 10 mM phosphate buffer (pH 7.2) to obtain a final concentration of 1mM p-nitrophenylacetate. To 1 mL of this solution, 1.9 mL of buffer and 100 μL of the tissue homogenate were added in a quartz cell. The contents were mixed by briefly inverting the cell and the kinetics of hydrolysis was evaluated at 348 mμ, using a Thermo Scientific

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GENESYS 6™ UV-Vis spectrophotometer, for 5 mins. For these studies, p-nitrophenyl acetate in the buffer solution, in the absence of the enzyme, was used as a blank.

HPLC Analytical Method

Samples were analyzed using an HPLC system consisting of a Waters 600 pump controller, a refrigerated Waters 717 plus autosampler, a 2475 Multi λ Fluorescence detector and a Agilent 3295 integrator. A Waters C18 Symmetry column, 4.6 × 250 mm, was used. Mobile phase for acyclovir analysis consisted of 15 mM phosphate buffer (pH 2.5) containing 1% acetonitrile. Detection was carried out at excitation wavelength of 270 nm and emission wavelength of 380 nm. Quinidine was analyzed at an excitation wavelength of 250 nm and a quantification wavelength of 440 nm, using a mobile phase consisting of 20 mM phosphate buffer (pH adjusted to 2.5) and acetonitrile (88:12).

Data Analysis

Flux and permeability calculations were performed as previously reported[23,27]. Briefly, steady state flux values were calculated from the plot of cumulative amount of drug in the receiver phase (C_{cum}) with respect to time (Eq. 1). Steady flux values were normalized to donor concentration (C_d) to calculate drug permeability (Eq. 2).

$$Flux\,(\mathrm{J}) = dC_{Cum}/dt \quad (1)$$

$$Permeability\,(\mathrm{P}_{app}) = Flux/\mathrm{C_d} \quad (2)$$

All experiments were carried out at least in triplicate. Student's t test for unpaired sample was used for statistical analysis. Data obtained for multiple groups was subjected to statistical analysis using One Way Analysis of Variance (ANOVA). Variation between the groups was checked using Levenes' test and significant difference between group means was determined using Tukeys HSD test. Results were considered statistically significant if p-value was < 0.05.

RESULTS

Light Microscopy

Hematoxylin-eosin stained cross sections for corneas extracted from ocular globes stored in PBS/HBSS did not show any significant difference from the fresh corneas (Fig. 1).

Trans-epithelial Electrical Resistance

TEER values represented in Fig. 2 for corneas from eyes preserved in PBS (4.7 ± 0.3 $K\Omega.cm^2$) and HBSS (4.8 ± 0.25 $K\Omega.cm^2$) were slightly lower but not significantly different from corneas from freshly isolated eyes (5.2 ± 0.3 $K\Omega.cm^2$). The TEER values remained constant throughout the three hour duration of the experiment.

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Paracellular and Transcellular transport

Permeability of the paracellular marker [^{14}C]mannitol was found to be approximately three times higher across corneas preserved in PBS or HBSS compared to freshly excised corneas ($3.96 \pm 1.44 \times 10^{-6}$ cm/sec). Ca^{+2} plays an important role in maintaining the integrity of tight junctions[28]. Since Ca^{+2} is present in HBSS it was thought that their presence would help maintain the integrity of the tight junctions. However, significant difference was not observed (Fig. 3) in the permeability of [^{14}C]mannitol between eyes preserved in PBS ($14.88 \pm 1.69 \times 10^{-6}$ cm/sec) and HBSS ($13.6 \pm 0.26 \times 10^{-6}$ cm/sec).

Corneal diffusion of acyclovir (Fig. 4), however, was not affected by the storage medium. Corneal permeability of acyclovir across corneas from fresh eyes ($3.25 \pm 0.11 \times 10^{-6}$ cm/sec) was not significantly different from those stored in PBS ($4.01 \pm 0.38 \times 10^{-6}$ cm/sec).

Permeability of the transcellular marker, [^{3}H]diazepam, in fresh corneas ($2.19 \pm 0.4 \times 10^{-5}$ cm/sec) was not significantly different from corneas extracted from eyes preserved in PBS ($2.35 \pm 0.59 \times 10^{-5}$ cm/sec) or HBSS ($2.59 \pm 0.34 \times 10^{-5}$ cm/sec)(Fig. 3). Also permeability of quinidine ($2.0 \pm 0.1 \times 10^{-5}$ cm/sec) was not affected when eyes were preserved in PBS ($2.5 \pm 0.4 \times 10^{-5}$ cm/sec) as depicted in Figure 4.

Amino Acid Transporter Activity

Eyes preserved in PBS and HBSS exhibited functional activity of the amino acid transporters. In the case of eyes preserved in PBS, transport of [^{14}C]L-Arginine (Fig. 5) across fresh eyes ($6.46 \pm 2 \times 10^{-6}$ cm/sec) was not significantly different from those obtained from eyes preserved in PBS ($6.59 \pm 2.2 \times 10^{-6}$ cm/sec) or HBSS ($7.06 \pm 1.68 \times 10^{-6}$ cm/sec). [^{14}C]L-Arginine transport was inhibited significantly by the specific L-amino acid transporter inhibitor, BCH, in eyes preserved in both PBS and HBSS (Fig.6A & B).

Corneal permeability of [^{14}C]phenylalanine ($9.89 \pm 0.49 \times 10^{-6}$ cm/sec, freshly excised corneas) was found to be significantly lower when the eyes were preserved in PBS ($6.9 \pm 0.54 \times 10^{-6}$ cm/sec). However, permeability values across the corneas from eyes that were preserved in HBSS ($9.45 \pm 0.67 \times 10^{-6}$ cm/sec) (Fig. 5) were not significantly different from the fresh corneas. In all cases transport of [^{14}C]phenylalanine was significantly inhibited in the presence of the inhibitor, BCH (Fig. 6A & B).

Active transport of [^{14}C]L-Alanine across corneas that were freshly excised ($1.87 \pm 0.35 \times 10^{-5}$ cm/sec) was found to be similar to those from eyes preserved in PBS ($1.57 \pm 0.90 \times 10^{-5}$ cm/sec) or HBSS ($1.99 \pm 0.15 \times 10^{-5}$ cm/sec)(Fig. 5). When the studies were carried out in sodium free medium permeability was significantly reduced (Fig. 6A & B) in all cases.

The observed corneal permeability values (from fresh eyes) of the amino acids are about 2 to 2.5 fold lower than literature reports [20-22]. This difference may be attributed to use of a different radiolabel (^{14}C instead of ^{3}H) being used in this study.

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Enzymatic Activity

Non-specific esterase activity was assessed using p-nitrophenyl acetate assay protocols. Esterase activity in preserved corneas was observed to be about 1.5-fold lower compared to the freshly excised corneas. There was, however, no significant difference in the esterase activity of corneas obtained from eyes preserved in PBS or HBSS (Table 1). Iris-ciliary tissues from eyes stored in PBS exhibited an almost 2-fold decrease in the esterase activity. However, esterase activity was preserved at normal levels in the iris-ciliary tissues when the eyes were stored in HBSS. The other ocular tissues tested did not demonstrate any significant difference between fresh or preserved eyes.

DISCUSSION

Drug molecules can permeate across the cornea by passive or active transport mechanisms. Passive diffusion across the cornea involves the paracellular or transcellular pathway. In transcellular diffusion the drug molecules partition into the lipoidal cell membrane, but permeability depends on the molecular size, charge and lipophilicity[29]. Paracellular diffusion, wherein the drug molecules move through the intercellular spaces, is limited by the tight junctions present on the superficial epithelial cells [30]. Several carrier systems, including the amino acid transporters ASCT1, LAT1 and $B^{o,+}$, have been reported on the corneal epithelium of rabbits [20,22,31]. These transporters are responsible for nutrient transport, but are being frequently targeted for drug delivery across biological barriers [31,32]. Thus, when transcorneal permeability of a therapeutic candidate is being evaluated *in vitro*, it is vital that these passive and active transport mechanisms remain unaltered in the model.

The objective of this study was to examine the utility of corneas isolated from preserved rabbit eyes for transcorneal permeability studies and to identify a media which would be economical, simple to prepare and would maintain the barrier and transport mechanisms of the cornea intact. A lot of work has been carried out in the field of preserving human corneas for transplantation. The cornea is either stored in organ culture media at physiological temperatures (31-37°C) or stored in serum free media at hypothermic temperatures (4°C)[33]. Organ culture media are expensive and calls for complex preparation steps. Moreover, the corneas have to be de-swelled in dextran before use. This would be a laborious process for preserving corneas for *in vitro* transport experiments. Hypothermic storage media are commercially available but are extremely expensive.

Spencer *et. al*. compared storage of isolated corneas in balanced salt solution(BSS) to McCarey Kaufman (MK) media for a period of four days in the presence or absence of the steroid hydrocortisone[34]. They reported that there was no significant difference if the eyes were stored in BSS or M-K media at 4°C. Also there was no significant difference on storage even in the presence of hydrocortisone. That study, however, was limited to the examination of autolysis using the marker enzyme, acid phosphatase.

In view of the earlier reports, HBSS was chosen as one of the storage media. Since the eyes were to be stored in this study for a relatively short duration (not more than 24 hours), PBS, the most common storage medium was also selected. Both HBSS and PBS buffers are easy to prepare, very economical and use chemicals readily available in most laboratories.

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Currently, two methods are used to transport corneal tissues. Either the whole eyeball is transported in a moist chamber, or, the cornea is isolated *in situ* and transported in a solution. *In situ* isolation has been reported to be better at preserving the cornea compared to transporting the whole eye globes[35]. The primary focus in preserving human corneas is to maintain endothelial cell viability and to prevent autolytic changes after death which may occur due to contact with stagnant aqueous humor. Thus, the cornea needs to be separated from the rest of the globe. However, for the purpose of drug permeation experiments the endothelium is a very weak barrier. Rather, an intact epithelium is essential for *in vitro* experiments.

TEER is an electrophysiological technique to measure the integrity of the paracellular pathway [36]. Although the TEER values of corneas excised from preserved eyes were observed to be higher compared to fresh corneas, the difference was not statistically significant, indicating no change in the epithelial barrier properties. However, TEER values alone cannot be used as a measure for assessing structural integrity. Permeability of [^{14}C]mannitol, a paracellular marker, was found to be three times higher in corneas from preserved eyes compared to freshly excised corneas. However, there was no significant difference in permeability of acyclovir, another hydrophilic molecule also known to permeate through the paracellular route[37]. The slightly lower TEER values and higher mannitol permeability of corneas from preserved eyes, compared to fresh corneas, indicates that the tight junctions are disrupted to an extent that small linear molecules like mannitol can pass through in greater quantities. A more branched molecular structure like acyclovir, however, shows no difference in corneal permeability in all models.

A significant alteration of the tight junction integrity would also have an impact on the permeability of lipophilic molecules which diffuse transcellularly. These lipophilic agents can diffuse through the paracellular route to a greater extent than in the presence of intact tight junctions. Lack of any changes in the transcorneal permeation values of the lipophilic, transcellular markers, diazepam and quinidine, indicates that the integrity of the junction proteins is not significantly disrupted, and permeability of only very small molecules, such as mannitol, is affected. Moreover, the observed transcellular permeability values for these lipophilic agents were statistically similar in the corneal tissues obtained from both fresh and preserved rabbit eyes.

Transporter targeted prodrugs have been designed to enhance transcorneal permeation of the parent moiety using a piggy-banking approach, where the prodrug is translocated by the carrier system. *In-vitro* transport experiments are thus often carried out to determine specific transporter involvement in the translocation process of a new therapeutic agent or to optimize a transporter targeted prodrug design approach. Preservation of the active transport mechanisms is thus essential for accurate permeation predictions or for the purpose of ranking various candidates. In this study we evaluated the permeability characteristics of three amino acid transporters reported on the rabbit corneal epithelium. Results indicate that the amino acid transporters, LAT1, ASCT1 and $B^{0,+}$, remain functionally active in the corneas extracted from the eyes preserved in PBS or HBSS as the presence of BCH or lack of sodium significantly decrease the transport of their substrates. However, for corneas extracted from whole globes preserved in PBS, the permeability of L-Phenylalanine was

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significantly lower compared to that across corneas from eyes that were freshly excised or were preserved in HBSS. Thus, corneas from rabbit eyes stored in HBSS exhibited functional activity of all three amino acid transporters at levels similar to that in freshly excised corneas. Both PBS and HBSS are isotonic but HBSS has added glucose and metal ions. Thus, even at low temperatures where metabolic activity is minimal some form of nutrition needs to be added to the medium for amino acid transporters to remain fully functional.

Esterase activity of corneas from eyes preserved in PBS and HBSS was observed to be lower compared to freshly excised cornea. The esterase activity in the other tissues, from eyes stored in HBSS, was not significantly different. The lower esterase activity in the cornea, compared to other tissues, can probably be explained by the fact that the cornea is exposed to the storage conditions (solution and period) to a greater extent in comparison to the other tissues which remain relatively protected inside the ocular globe, deriving nutrition from the vitreous humor. The reduced enzyme activity may impact bioreversion and thus permeability rates. However, considering the extremely rapid enzymatic degradation rates observed with most ester prodrugs and the low drug concentrations used in general, the impact of this 1.5-fold reduction in enzymatic hydrolysis rate may not be very significant with respect to relative permeability evaluation.

In conclusion, the results from this study indicates that storage of the extracted whole rabbit eye in HBSS, over wet ice, for a period of up to 24 hours, preserves the active and passive transport mechanisms in the cornea. Eyes shipped from abattoirs in this manner, within this timeframe, would serve as an excellent *in vitro* model for transcorneal permeation studies and would significantly decrease the need for laboratory animal sacrifice. Further studies evaluating means to extend the period of storage are currently underway.

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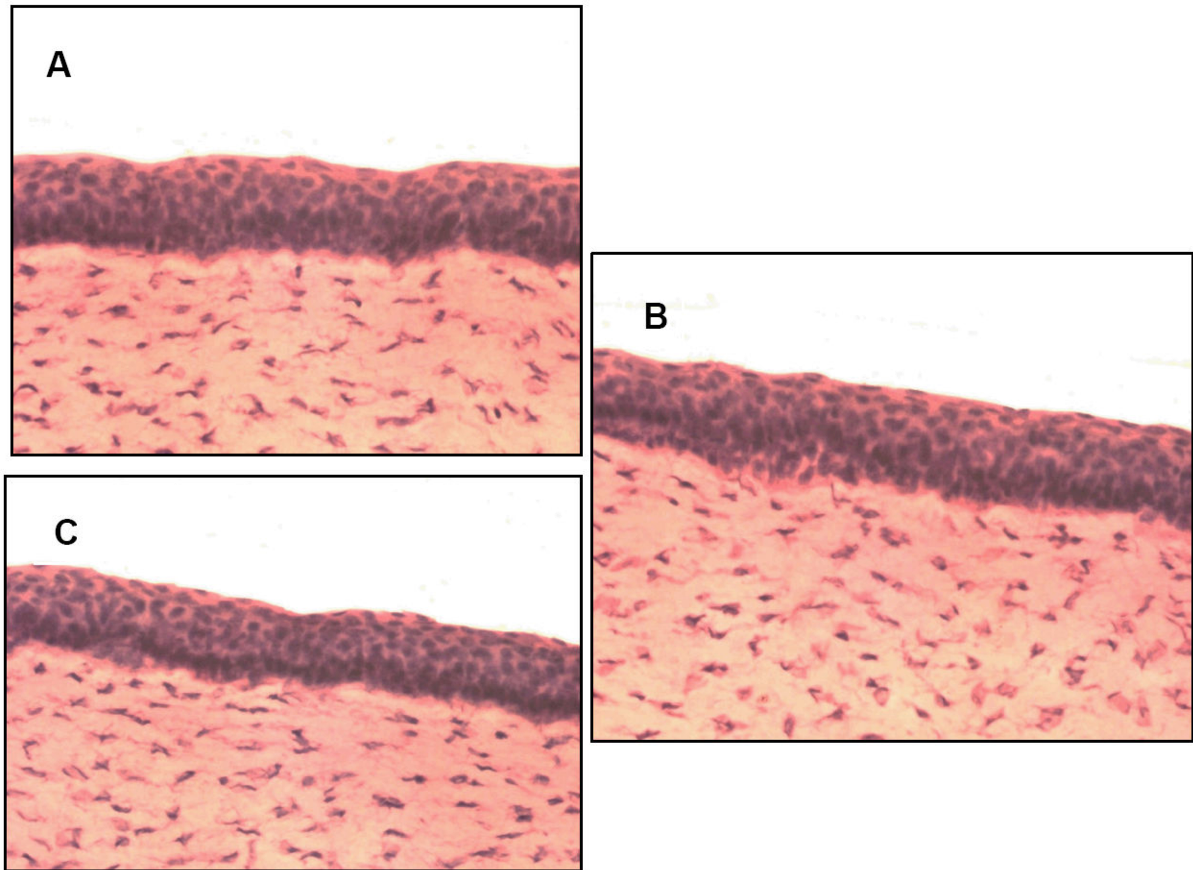

Figure 1.
Hematoxylin-eosin stained cross section of rabbit cornea. (A) Freshly excised rabbit cornea. (B) Cornea extracted from eyes preserved in PBS over wet ice for 24h. (C) Cornea extracted from eyes preserved in HBSS over wet ice for 24 hours.

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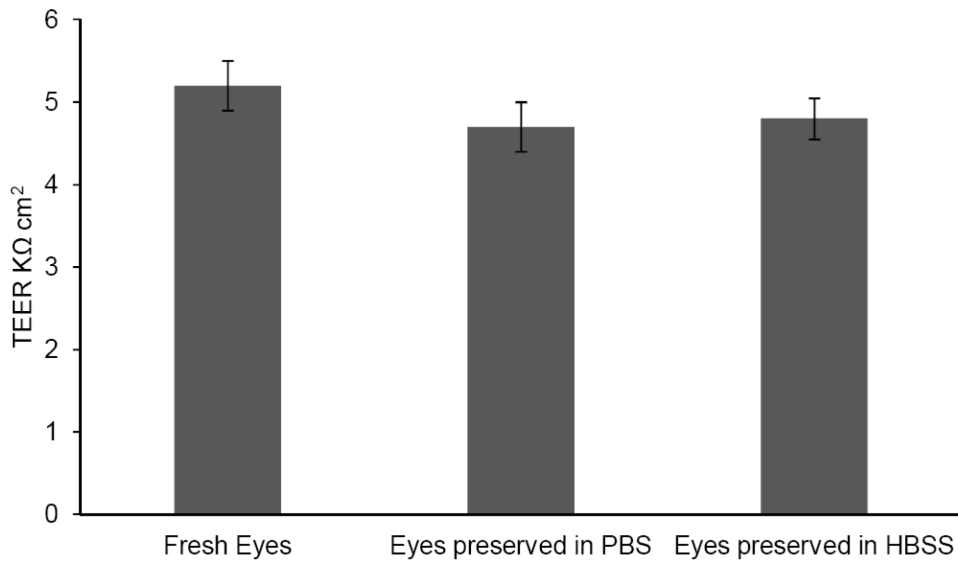

Figure 2.

Trans-epithelial electrical resistance (TEER) values across freshly excised corneas and corneas obtained from eyes preserved in PBS/HBSS over wet ice for 24h. Results are depicted as mean ± S.D (n=4).

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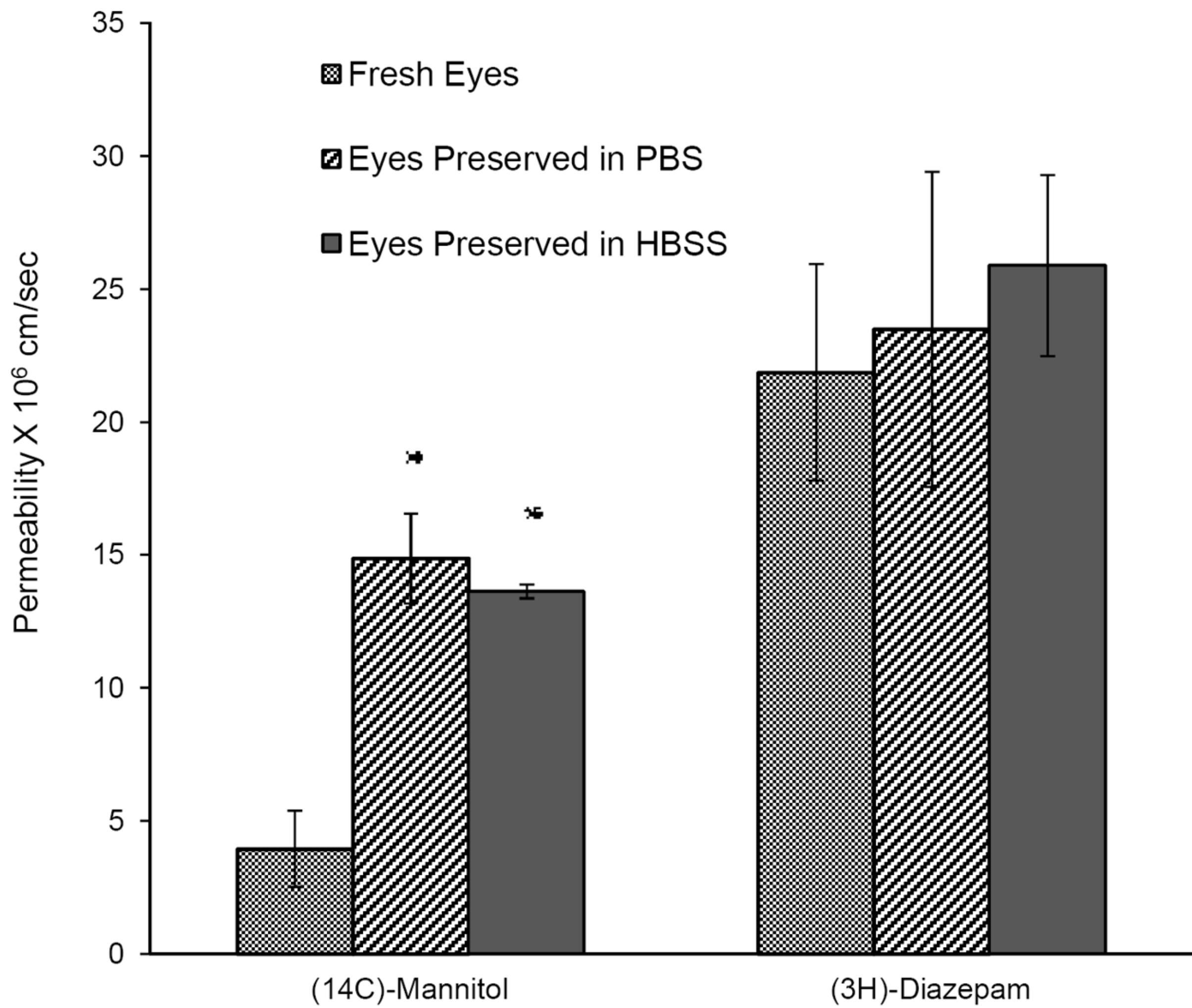

Figure 3.

Transcorneal permeability of [^{14}C]Mannitol (0.5 µCi/ml) and [^{3}H]Diazepam (0.5 µCi/ml), across corneas from fresh or preserved (in PBS or HBSS over wet ice for 24h) rabbit eyes. Results are depicted as mean ± SD (n=3). * $p<0.05$

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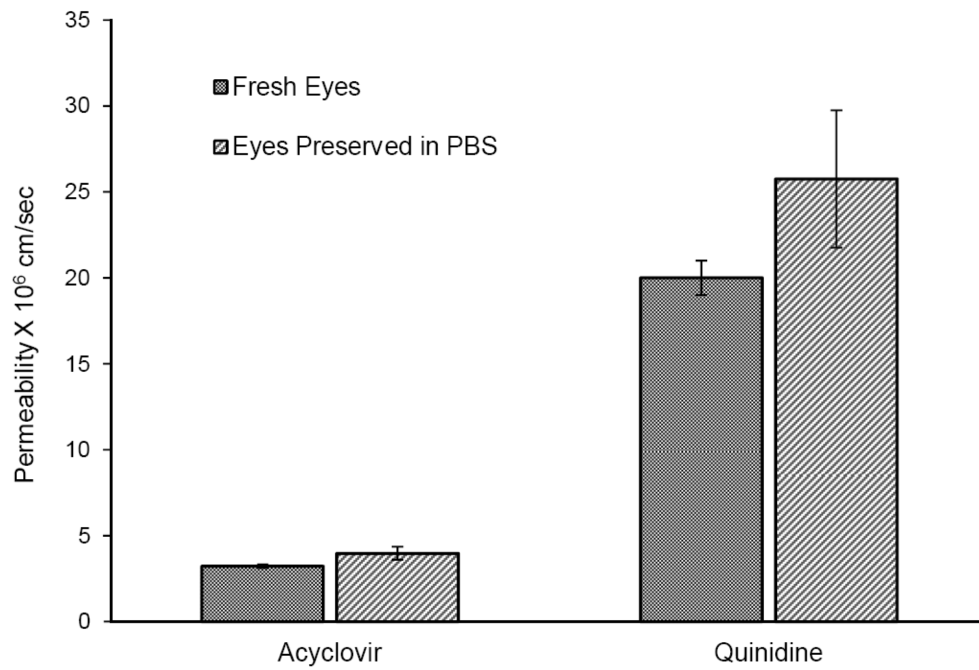

Figure 4.

Transcorneal permeability of acyclovir and quinidine across corneas from eyes preserved in phosphate buffered saline (PBS) over wet ice for 24h compared with freshly excised rabbit corneas. The studies were conducted at 34°C. Results are depicted a mean ± SD (n=3). * $p<0.05$

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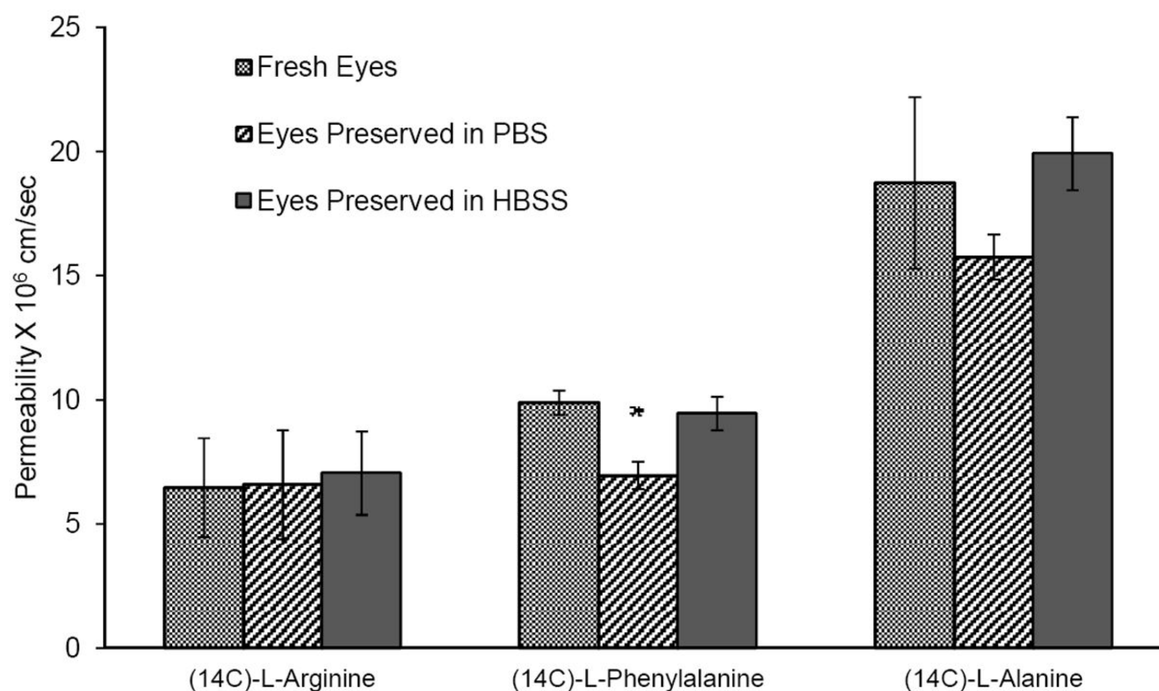

Figure 5.

Transcorneal permeability of [^{14}C]L-Arginine (0.5 μCi/ml), [^{3}H]L-Phenylalanine (0.5 μCi/ml) and [^{14}C]L-Alanine (0.5 μCi/ml) across corneas from fresh or preserved (in PBS or HBSS over wet ice for 24h) rabbit eyes. Results are depicted as mean ± SD (n=3). * $p<0.05$

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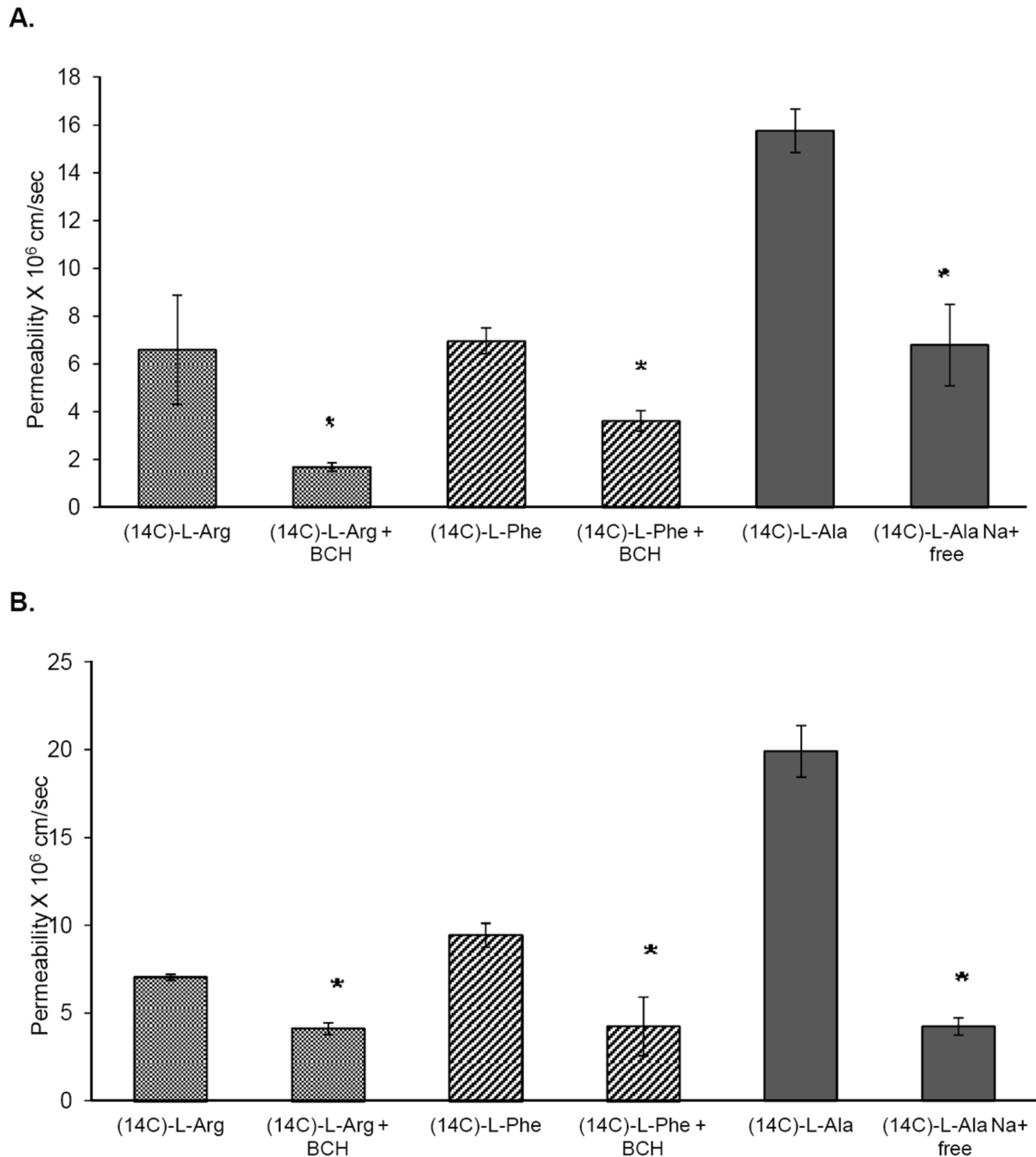

Figure 6.

Permeability of [^{14}C]L-Arginine (0.5 μCi/ml), [^{14}C]L-Arginine (0.5 μCi/ml) in the presence of BCH (5mM), [^{14}C]L-Phenylalanine(0.5 μCi/ml), [^{14}C]L-Phenylalanine(0.5 μCi/ml) in the presence of BCH (5mM), [^{14}C]L-Alanine (0.5 μCi/ml) and [^{14}C]L-Alanine (0.5 μCi/ml) in Na^+ free medium, across corneas from eyes stored for 24h in A) phosphate buffered saline (PBS) and B) hanks balanced salt solution (HBSS) over wet ice, at 34°C. Results are depicted as mean ± SD (n=3). * $p<0.05$

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Table 1

Esterase activity in fresh eyes compared to eyes preserved in PBS/HBSS over wet ice for 24h using p-nitrophenyl acetate as substrate. Results are depicted as a mean ± SD (n=3).

	Rate of hydrolysis of p-nitrophenol acetate [μM/(min.mg protein)]		
	Fresh Eyes	**Eyes Preserved in PBS**	**Eyes Preserved in HBSS**
Cornea	2.78 ± 0.19	*1.79 ± 0.11	*1.79 ± 0.11
Aqueous Humor	2.59 ± 0.19	3.09 ± 0.39	2.78 ± 0.32
Iris-Ciliary Body	14.01 ± 0.11	*8.46 ± 0.11	13.77 ± 0.11
Vitreous Humor	4.26 ± 0.19	5.06 ± 0.11	4.63 ± 0.19
Retina-Choroid	3.33 ± 0.19	4.51 ± 0.11	4.51 ± 0.11

* $p<0.05$