Université de Paris
**École doctorale Hématologie-Oncogenèse-Biothérapies (ED561)**
***Laboratoire Biologie, ingénierie et imagerie de la Greffe de Cornée***
***EA 2521***

# Modèle de conservation ex vivo de l'épithélium cornéen

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Thèse de doctorat de Biologie Moléculaire et Cellulaire
Spécialité : Biothérapies

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Présentée et soutenue publiquement le 11/10/2019

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**Titre : Modèle de conservation ex vivo de l'épithélium cornéen**

**Résumé :**

La cornée est la partie transparente située à l'avant de l'œil, constituée de trois types cellulaires principaux (cellules épithéliales, kératocytes et cellules endothéliales) en interaction pour garantir l'homéostasie tissulaire. Les modèles utilisés pour étudier *ex vivo* la cicatrisation sur cornées humaines ou animales ne permettent pas une conservation prolongée et documentée de la cornée en homéostasie. Un tel modèle est pourtant essentiel pour évaluer rapidement la toxicité ou l'intérêt de nouvelles molécules sans avoir recours systématiquement à l'expérimentation animale. Une machine de conservation active (MCA ; aussi appelé bioréacteur (BR)) a été développée pour imiter en laboratoire les conditions physiologiques de la cornée. L'objectif était d'évaluer l'effet du bioréacteur sur la conservation de cornées porcines et humaines, notamment sur l'épithélium de cornées. Comparé aux systèmes passifs, la MCA permet de conserver des cornées porcines pendant 7 jours ; utilisée avec des cornées humaines, la MCA permet d'améliorer la préservation et la différenciation de l'épithélium de cornées humaines préalablement conservées en organoculture. Cette réhabilitation les rend utilisables pour l'expérimentation ex vivo.

**Mots clefs :**

Organoculture ; Greffe de cornée ; Cicatrisation cornéenne ; Modèle animal ; Bioréacteur .

**Title : Ex vivo conservation model of the corneal epithelium**

**Abstract :**

The cornea is the transparent part located at the front of the eye, made up of three main cell types (keratinocytes, keratocytes and endothelial cells) interacting to guarantee tissue homeostasis. Current ex vivo models used to study wound healing on human or animal corneas do not enable prolonged and documented preservation of the cornea in homeostasis. However, such a model is essential to quickly evaluate the toxicity or the interest of new molecules without using systematically animal experimentation. An active storage machine (ASM; also called bioreactor (BR)) has been developed to mimic the physiological conditions of the cornea in the laboratory. The objective was to evaluate the effect of the ASM on the conservation and differentiation of porcine and human corneas, particularly on the corneal epithelium. Compared to passive systems, this innovative system enables to preserve pig corneas for 7 days; when used with human corneas previously stored in organ culture, the ASM improves the preservation and differentiation of the epithelium.

**Keywords :**

Eye banking ; Corneal graft ; Epithelial wound healing ; Animal model ; Bioreactor.

# Remerciements

A mes Maîtres,

Je remercie Monsieur le Professeur Éric Gabison, pour ton compagnonnage, ton inépuisable esprit créatif et critique, ton enthousiasme et ta patience. Merci de m'avoir permis d'avoir une expérience si variée, de me guider tout en prenant le temps d'écouter. La route est encore longue, riche en échanges et en projets (et sushi/pizza).

Je remercie Monsieur le Professeur Gilles Thuret, pour m'avoir accompagné, commenté et pour toujours ajouter de nouvelles idées pour m'aider à m'améliorer, faire évoluer les projets pour viser le meilleur et pourquoi pas ce qui parait impossible pour le dépasser un jour.

Je remercie Monsieur le Professeur Philippe Gain pour m'avoir accueilli et accompagné depuis mes débuts en laboratoire, il y a plusieurs années déjà. Grand modèle de chercheur-trouveur, vous avez toujours pris le temps pour moi et fait en sorte que ce projet puisse continuer. Merci & allez les verts !!!

Je vous remercie tous les trois pour votre humanité, votre humilité, votre enthousiasme, mais aussi pour les moments de convivialité qui ont jalonné ces années. Vous incarnez un idéal qui m'aura influencé et continuera de m'influencer. Vous êtes l'incarnation du rêve de cette carrière mixte partagée entre médecine, sciences et enseignement.

Je remercie Messieurs les Professeurs Fréderic Chiambaretta et David Touboul pour leur relecture et participation à ce jury en tant que rapporteurs. J'espère être amené à mieux vous connaître dans le futur.

Je remercie Madame la Professeure Samia Mourah pour m'avoir accueilli dans votre laboratoire à mes débuts, ainsi que pour votre participation à ce jury.

Je remercie Monsieur le Docteur Emmanuel Crouzet, pour tout le chemin parcouru, ta persévérance et ton amitié. Mais aussi, de faire rayonner les verts ! La distance se réduisant j'ai hâte de pouvoir re-partager des moments de convivialité qui me sont chers.

J'ai une reconnaissance infinie envers le laboratoire BiiGC, ses meneurs, ses membres et une grande fierté de faire partie de ce fleuron Français de l'innovation et de la formation des futurs chercheurs. Je remercie tous ses membres, pour l'aventure passée mais également futures. Chaleureux remerciements à Chantal, Simone, Zhiguo, Clotilde, Émilie, Aurélien, Pascal, Damien.

A Mélanie Marie, indéfectible hotline & psychothérapeute pour doctorant. Merci d'avoir partagé ton expérience, toujours répondu à mes innombrables questions. J'ai encore d'autres rhum à te faire découvrir !

Aux financeurs, la Fondation pour la recherche médicale, la Fondation des gueules cassées, la Fondation Rothschild, l'Université d'Harvard, la commission franco-américaine Fulbright, la Fondation Philippe.

A la mémoire des donneurs.

Vous cherchiez vraiment cette page ?

A mes parents, ma sœur, Vincent, Jules César & Émilie

A Adèle,

A mes amis,

Aurore, Romain, Nobel, MLTM, Cécile, Guillaume, Nico, Jess, 139, Le vice du vice, Thomas, Ninon, Bobok, Krikri, Geoffroy, Sarah, Mickael, Claire, Elsa, Antoine, Camille, Julie, Cam, Clémence (& ?), Jozu, Emilien, Cha, Esther, Marie Alice, Audrey, Marie Sarah, Mayssa, Aurélien, Morgane, Thomas, Mathilde, Pauline, Bubu, Vael, Emilie, Sarah, Sara, Sirine, Pierre

La team Amérique, Clotilde, Will, Jen, Hannah, Zala, Yukako, Giulia, Maria, Dina, Ashley, Liv, Panu, Mads

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# 1. Introduction

La cornée est la partie transparente située à l'avant de l'œil. Elle joue un rôle essentiel pour la vision. Les pathologies aigües ou chroniques de la cornée et de la surface oculaire constituent un motif de consultation fréquent en ophtalmologie. Pour offrir des réponses, la recherche fondamentale et appliquée se sont développées afin de comprendre les différents mécanismes physiopathologiques impliqués et tenter de développer de nouvelles solutions thérapeutiques. Ces développements requièrent de tester les dispositifs ou les molécules sur des modèles avant d'envisager de les étudier chez l'Homme. Cette phase de criblage et de test peut être difficile à mettre en œuvre, coûteuse, et la généralisation des résultats à l'Homme peut être difficile dans le cas d'utilisation de modèles animaux. Pour faciliter ces démarches, le laboratoire de Biologie, Ingénierie et Imagerie de la Greffe de Cornée (BiiGC) développe une plateforme expérimentale (bioréacteur ou machine de conservation actif) capable d'accueillir des cornées d'origine humaine ou animale. Avant d'envisager toute expérimentation, une telle plateforme doit pouvoir assurer une conservation des cornées en homéostasie, tâche rendue difficile de par les caractéristiques physiologiques de la cornée et les différents types cellulaires qui y cohabitent. L'objet de cette thèse était de potentialiser les capacités du bioréacteur pour la conservation *ex vivo* de l'épithélium de cornées porcines puis humaines. Les futurs travaux porteront sur la validation du bioréacteur en tant que plateforme expérimentale pour la réalisation de tests pré-cliniques (toxicité, cicatrisation, etc.).

## 1.1. La cornée

La cornée assure une fonction réfractive grâce à sa transparence et à sa géométrie qui lui confère les 2/3 de la puissance dioptrique de l'œil. En interaction avec le milieu extérieur, la cornée est exposée à l'environnement, aux traumatismes accidentels et de plus en plus aux traumatismes chirurgicaux, notamment dans le cadre de la chirurgie réfractive[1]. La cornée peut aussi être atteinte par des pathologies qui lui sont propres (dystrophie) ou être atteinte par des pathologies systémiques.

### 1.1.1. Structure et fonction

Chez l'Homme, la cornée est constituée de 5 couches : un épithélium, la couche de Bowman, le stroma, une membrane de Descemet et d'un endothélium. Il s'agit d'un tissu avasculaire, en interaction avec l'air, les larmes et la paupière en antérieur, avec l'humeur aqueuse en postérieur.

L'épithélium cornéen est stratifié, squameux, non kératinisé, composé de 4 à 6 couches cellulaires et est d'une épaisseur d'environ 50µm. Il joue un rôle de barrière grâce à des jonctions serrées entre les cellules les plus superficielles et au glycocalyx situé à sa surface. Les cellules basales de l'épithélium reposent sur une membrane basale, permettant leur ancrage et la régulation des interactions entre les cellules épithéliales et les cellules stromales. Le renouvellement de l'épithélium cornéen est assuré par des cellules souches localisées au limbe[2,3].

Le stroma cornéen est composé de fibres de collagène hautement organisées, de protéoglycans et de cellules stromales (kératocytes). L'organisation du stroma est critique pour la transparence de la cornée. Cette organisation est influencée par la composition de la matrice extracellulaire, qui peut être perturbée en cas de dépôts (cicatrice par exemple), et par l'état d'hydratation du stroma, en grande partie régulée par l'endothélium cornéen.

L'endothélium cornéen est une monocouche cellulaire non régénérative dont la fonction est de maintenir le stroma cornéen dans un état de déshydratation relative, grâce à des pompes ioniques, permettant de maintenir l'organisation des fibres de collagène du stroma.

### 1.1.2. Homéostasie cornéenne

#### 1.1.2.1. Compartiment endothélio-stromal

Pour expliquer la régulation de l'état d'hydratation du stroma cornéen, Bryant et al.[4] ont proposé une relation liant la pression d'imbibition (PI), la pression intraoculaire (PIO) et la pression de gonflement (PG) : PI=PIO-PG. La pression d'imbibition est la pression exercée sur la cornée empêchant la survenue d'un œdème cornéen, la pression de gonflement est liée à la répulsion électrostatique des glycosaminoglycanes du stroma induisant un espacement des fibres de collagène et un appel d'eau. La pression intraoculaire s'oppose donc à l'appel d'eau vers le stroma lié aux glycosaminoglycanes. Cette équation suggère le rôle de la pression

intraoculaire dans la régulation de l'état d'hydratation de la cornée ; cette modélisation simple est bien connue des cliniciens, au travers des exemples classiques de grande hypotonie après chirurgie filtrante ou d'hypertonie post kératoplastie.

Cet état d'hydratation est également régulé par les cellules endothéliales, grâce à la présence de différentes pompes, permettant de réguler les flux d'eau.

La barrière épithéliale intervient également, un œdème cornéen peut être observé en regard d'une zone de disruption de l'épithélium cornéen.

#### 1.1.2.2. Épithélium cornéen

L'épithélium cornéen est en renouvellement permanent[5] et joue un rôle majeur dans le maintien de la transparence de la cornée. La cornée est avasculaire et l'environnement des cellules épithéliales est déterminant pour leur croissance et leur différenciation. Les larmes et les nerfs cornéens jouent un rôle trophique en apportant des facteurs de croissance essentiels à la survie des cellules épithéliales. L'exposition à l'air de l'épithélium est également impliquée dans la trophicité et la stratification des cellules épithéliales ; cette exposition peut être reproduite en culture par une technique dite d'« airlifting »[6] qui mime l'effet de l'évaporation du film lacrymal.

L'épithélium cornéen semble également être en interaction avec l'endothélium cornéen. Ceci est suggéré *in vitro* par l'observation d'une modification de l'assemblage de la membrane basale épithéliale lors de la co-culture de cellules endothéliales et épithéliales cornéennes[7]. Les épithéliopathies symptomatiques sont également bien connues des cliniciens en cas de dysfonction endothéliale[8]. Chez des patients affectés par une kératopathie bulleuse (liée à une dysfonction endothéliale), une diminution de la stratification épithéliale, des anomalies de la membrane basale épithéliale ainsi qu'une réduction de l'expression de protéines de surface (MUC16) ont été décrites[8].

Les cellules épithéliales cornéennes sont en interaction avec les cellules du stroma (kératocytes) à l'état physiologique comme pathologique[9]. En homéostasie, ces cellules échangent des facteurs de croissance (HGF, KGF[10] par exemple). En présence d'une lésion épithéliale avec ou sans altération de la membrane basale, les interactions épithélio-stromales sont modifiées[11]. Des cytokines et des facteurs de croissance sont synthétisés et sécrétés par les cellules épithéliales à destination du stroma et en retour, des facteurs de croissances sont

synthétisés par les kératocytes du stroma, ce qui influence la cicatrisation épithéliale[9]. Ces interactions épithélio-stromales sont dites « indirectes » lorsqu'elles sont médiées par des facteurs solubles (Transforming Growth Factor ß, Interleukine-1, par exemple). En cas de retard de cicatrisation épithéliale, ces interactions épithélio-stromales indirectes caractérisées alors comme « pathologiques » peuvent générer une fibrose du stroma par une synthèse et une accumulation de matrice extracellulaire par les kératocytes. La cicatrice générée peut être responsable d'une baisse d'acuité visuelle. Des interactions épithélio-stromales directes, impliquant des contacts cellulaires, ont également été décrites en cas d'ulcérations[11]. Ainsi, la présence de ces interactions épithélio-stromales directes peut à l'inverse des précédentes entrainer une fonte stromale, via CD147 (EMMPRIN), et l'induction de métalloprotéinases (MMP) pouvant aller jusqu'à une perforation cornéenne[11] et, en l'absence de traitement, à la perte du globe.

Les cellules souches épithéliales sont supposées présentes au niveau du limbe, parmi les cellules basales des palissades de Vogt. Il n'existe pas de marqueur spécifique connu des cellules souches, en revanche certains antigènes sont associés à des cellules peu différenciées (ABCB5[12], ΔNp63α[13] par exemple) alors que d'autres sont associés à des cellules épithéliales différenciées (K3/K12)[14].

### 1.2. Modèles expérimentaux étudiant l'épithélium cornéen

La démarche expérimentale requière des modèles pour reproduire des situations physiologiques et pathologiques afin de développer les connaissances et tester de nouvelles hypothèses ou molécules. Ce préalable est généralement nécessaire avant de réaliser tout test chez l'homme.

Comme souligné au chapitre précédent, la cornée est un tissus complexe, comprenant plusieurs types cellulaires avec des interactions multiples et une régulation fine. La création de modèles pour étudier la cornée en laboratoire est donc un tâche difficile. Tous les modèles expérimentaux ont des avantages et des limitations qu'il ne faut pas ignorer, afin de connaître les limites de la généralisation des résultats obtenus. Ci-après, nous dressons un panorama des différents modèles utilisés pour l'étude des cellules épithéliale de la cornée.

### 1.2.1. Modèles *in vitro*

Les modèles les plus simples utilisent des cellules épithéliales isolées et cultivées *in vitro*. Ces modèles peuvent être particulièrement utiles pour réaliser du criblage de molécules. Ils ont également l'avantage de pouvoir utiliser des cellules d'origine humaine.

Cependant, plusieurs éléments sont à prendre en considération :

- Les modifications phénotypiques des cellules selon les conditions de culture (support, milieu de culture, etc.)
- La perte des interactions entre les cellules épithéliales et les autres types cellulaires (kératocytes, cellules endothéliales, cellules immunitaires) ou avec les structures de voisinage (nerfs cornéens)
- Une senescence rapide en l'absence de sélection et de conditions de culture particulières

En culture, les cellules épithéliales forment une monocouche cellulaire ou s'organisent de manière pauci-stratifiée avec une formation limitée des jonctions serrées et une absence de barrière épithéliale, soit un tissu avec une structure éloignée de l'épithélium *in situ*[15,16]. Pour tenter de former un tissu proche de l'épithélium cornéen in situ, des modèles plus complexes ont été élaborés, utilisant des supports (membrane amniotique[17], par exemple) ou plaçant les cellules dans des conditions particulières (exposition à l'air[18], ajout de calcium[19], exposition au sérum[15]) permettant d'induire une stratification et une expression des jonctions serrées apicales et de restaurer partiellement la fonction de barrière épithéliale. Néanmoins, ces systèmes ne permettent pas de restaurer l'ensemble des interactions se produisant in situ (nerfs cornéens, cellules immunitaires, etc.).

Ce genre de modèle n'est pas utilisable pour la mise en place de modèles de chirurgie de la cornée (chirurgie réfractive par exemple).

### 1.2.2. Modèles *in vivo*

Les modèles animaux sont les modèles les plus complexes pour l'expérimentation. Ils sont nécessaires pour la validation de molécules avant de réaliser des essais chez l'homme. Les espèces utilisées sont multiples, du poisson au mammifère, chacun avec un degré variable de

similarité avec l'Humain. Cependant, de nombreuses limitations doivent être prises en compte:

- Contraintes éthiques. La règle des 3R (remplacer, réduire, raffiner) pousse à une réduction de l'expérimentation animale et à limiter son recours quand aucune alternative existe.
- Contraintes organisationnelles. L'expérimentation animale requière une animalerie, avec des infrastructures et un personnel adaptés aux modèles utilisés, et au protocole qui doit être réalisé (bloc opératoire, par exemple).
- Contraintes administratives. La mise en place d'un protocole d'expérimentation animale requiert la soumission et la validation de ce protocole à un comité d'éthique, imposant des délais incompressibles avant de démarrer un protocole.
- Contraintes financières. Les coûts associés à la réalisation d'un protocole d'expérimentation animale peuvent être importants (achat des animaux, hôtellerie, matériels et médicaments dédiés, prestations diverses, etc.).
- Contrainte de débit. Le temps dédié est généralement important. Couplé aux autres contraintes (éthiques, coût, réglementaires, etc.), un protocole d'expérimentation animale n'est pas adapté au criblage à haut débit.
- Limitation pour la généralisation des résultats. Des différences notables en terme d'anatomie, de physiopathologie, de biodisponibilité peuvent être observées entre l'homme et les différents modèles animaux disponibles[20]. Ces limitations sont à prendre en compte lors de la planification du protocole d'expérimentation animale et lors de l'interprétation et de la généralisation des résultats.
- Contrainte sociale. La vision du public concernant l'expérimentation animale peut être négative, particulièrement pour certains modèles (chiens, chats, singes, etc.).

### 1.2.3. Modèles *ex vivo*

Les modèles *ex vivo* peuvent constituer une solution alternative. Il s'agit de prélever le globe ou la cornée et de les placer dans des conditions de culture adaptées en laboratoire. Parmi les avantages, on note :

- La disponibilité des tissus :

    - En grande abondance pour les espèces communes (porc, bovin par exemple)
    - Possibilité de disposer de tissus humains dans un cadre règlementaire particulier
    - Possibilité d'obtenir des tissus avec une structure proche de celle observée *in vivo*, en collectant les tissus après un délai post mortem court
- La possibilité de réaliser les expériences dans n'importe quel laboratoire en recourant au matériel usuel (incubateur), sans nécessité de recours à une animalerie
- La réduction des coûts du fait de l'absence de recours à l'animalerie
- La possibilité de conserver la structure tridimensionnelle de la cornée

Dans le cas de la cornée, quelques inconvénients peuvent être soulignés :

- Absence d'innervation
- Absence de relation avec le système vasculaire et immunitaire
- Possibilité de conservation limitée dans le temps
    - Risque d'infection
    - Dégradation des tissus en l'absence de conditions de conservation optimisées
- Modification des interactions avec les structures adjacentes (paupières, air par exemple)

Les modèles les plus simples pour stocker les cornées ou réaliser de l'expérimentation *ex vivo* sont dits « passifs » et placent la cornée dans une boite de Petri, immergée dans du milieu de culture ou en « air lifting ». Il s'agit du modèle le plus commun, utilisé actuellement par les banques de cornées, ou pour les premiers modèles de cicatrisation ou de toxicité *ex vivo*. Cependant, cette méthode ne permet pas une conservation prolongée de la cornée. Les investigateurs se sont alors portés vers des solutions dites « actives » pour améliorer la conservation des cornées. Nous détaillons au prochain chapitre cette évolution.

# 2. Le bioréacteur ou machine de conservation active pour la cornée

## 2.1. Contexte

### 2.1.1. La greffe de cornée

Une greffe de cornée est indiquée en cas de perte de transparence de la cornée (fibrose stromale, dystrophies, etc.), de déformation de la cornée (kératocône, etc.) ou d'œdème cornéen lié à une perte excessive en cellules endothéliales (dystrophie endothéliale de Fuchs, traumatisme chirurgical, etc.).

La greffe de cornée a longtemps été synonyme de kératoplastie transfixiante, technique remplaçant la cornée centrale du receveur avec la cornée centrale issue d'un receveur. Désormais, grâce aux évolutions récentes des techniques de greffe de cornée, il est généralement possible de remplacer de manière élective le stroma ou l'endothélium cornéens, en réalisant une kératoplastie lamellaire antérieure (KLA) ou postérieure (KLP), respectivement. L'épithélium n'est pas remplacé au cours des techniques de kératoplastie sus-décrite, sa greffe est l'objet de techniques spécifiques de reconstruction de la surface oculaire.

Pour réaliser ces techniques de kératoplastie, du tissus issus d'un donneur cadavérique est nécessaire. Initialement, le tissus était prélevé puis immédiatement greffé, il s'agissait de la « greffe fraîche ». Cependant, les contraintes sanitaires, le contrôle qualité des tissus greffés et l'organisation du processus de greffe ont imposé d'organiser la conservation des greffons pour donner le temps nécessaire à la réalisation de ces étapes. Cette étape de conservation ex vivo des tissus est appelée « organoculture » ou « conservation hypothermique » selon la technique utilisée. L'enjeu de la conservation des cornées est tourné vers une meilleure conservation des cellules endothéliales qui ne sont pas régénératives et conditionnent le pronostic des kératoplasties endothéliales et transfixiantes. Les cellules épithéliales sont mal conservées en organoculture standard[21] et le greffon une fois implanté est colonisé par l'épithélium du receveur dans le cas des kératoplasties transfixiante ou lamellaire antérieure.

### 2.1.3. L'organoculture

La technique d'organoculture des cornées humaines n'a pas changé depuis les années 1970[22,23]. La cornée du donneur est prélevée avec une collerette de sclère, déposée dans un conteneur hermétique contenant du milieu de culture, puis conservée en étuve à 31-37°C (température variable selon les écoles). Le milieu de culture comprend du Minimal Essential Medium (MEM), supplémenté avec du sérum de veau fœtal[24]. Les cornées sont ainsi conservées jusqu'à 5 semaines. Pendant cette période, la cornée devient œdémateuse[25] en raison de la disparition de la pression intra oculaire exercée normalement sur le stroma et d'une perte partielle et temporaire de fonction de la pompe endothéliale. Pour limiter cet œdème avant la greffe, la cornée doit être exposé à des agents osmotiques 24 à 72h avant greffe (dextran ou poloxamer). Cependant, l'effet n'est que partiel et l'œdème n'est totalement résorbé qu'en quelques semaines chez le patient, retardant la récupération visuelle. De plus, le dextran est toxique pour les cellules endothéliales[26]. L'œdème cornéen induit des plis postérieurs où les cellules endothéliales meurent de façon prépondérantes[27]. Or, la survie des cellules endothéliales conditionne en partie la durée de vie du greffon cornéen.

### 2.1.4. Conservation hypothermique

Les cornées sont placées dans un milieu de culture puis sont stockées dans un réfrigérateur à une température comprise entre 2–6°C. La conservation est généralement de 5 à 10 jours[22]. Les altérations de l'endothélium et de l'épithélium tel qu'observées en organoculture sont similaires en conservation hypothermique.

## 2.2. Changement de paradigme

Les techniques de conservation des cornées utilisées jusqu'à présent et sus décrites sont des techniques dites « passives », plaçant la cornée dans un environnement éloigné de celui qui leur permet de survivre pendant des dizaines années. Dans ces conditions, le greffon cornéen s'altère (perte des cellules endothéliales, perte de l'architecture stromale, perte de l'épithélium cornéen).

L'équipe du laboratoire Biologie, Ingénierie et Imagerie de la greffe de cornée (BiiGC, EA2521) a développé une machine de perfusion permettant une conservation « active » des cornées.

L'objectif est de reproduire des conditions de conservation aussi proche que possible de la physiologie, afin de promouvoir la préservation des cellules endothéliales (objet du travail de thèse du Dr Thibaut Garcin[28]), du stroma et de l'épithélium cornéen.

### 2.3. Fonctionnement du bioréacteur

Afin de tenter de prendre en compte les divers principes de physiologie explicités, différents choix pragmatiques ont été réalisés. En premier lieu, le bioréacteur avait pour but d'améliorer le fonctionnement du couple endothélium-stroma. La première solution mise en place visait à restaurer un équivalent de pression intraoculaire et d'assurer un renouvellement du milieu de culture afin de limiter l'œdème stromale et de limiter la perte en cellules endothéliales. Pour recréer un gradient de pression entre le compartiment endothélial et le compartiment épithélial tout en renouvelant le milieu de culture, certains principes de chambre antérieur artificielle perfusée ont été repris avec des innovations probantes.

L'anneau scléral permet d'ancrer la cornée à la base du bioréacteur et ainsi de séparer le compartiment épithélial du compartiment endothélial (**Figure 1**). La pression est recrée dans le compartiment endothélial grâce à une pompe péristaltique. Le milieu de culture est renouvelé régulièrement. Le bioréacteur est usiné dans divers matériaux biocompatibles. En raison des différences de taille et de morphologie de la cornée porcine, une version spéciale du bioréacteur a été conçu pour les accueillir ; les autres principes restent par ailleurs identiques.




**Figure 1 – Principes du bioréacteur.**

Prototype du bioréacteur en fonctionnement, connecté au circuit de stockage et de renouvellement du milieu de culture. Le compartiment endothélial du bioréacteur était rempli avec du milieu d'organoculture (contenant du rouge phénol comme indicateur de pH (2)) maintenu à une pression de 20mmHg. Ici, le compartiment épithélial était laissé temporairement vide pour réaliser des mesures optiques en simulant des conditions physiologiques. La circulation du milieu d'organoculture était assuré par une pompe péristaltique (3) contrôlée par un capteur de pression (4), une électrovanne (5) et un microcontrôleur (6).

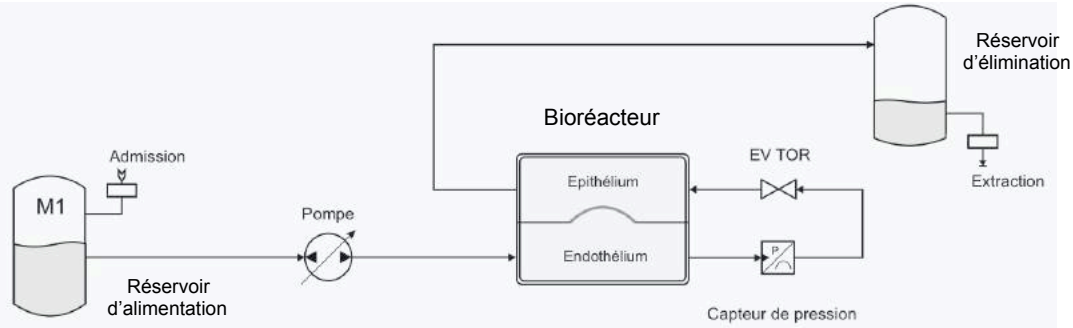



**Figure 2. Schéma fonctionnel du système bioréacteur immergé.**

EV TOR : Electrovanne Tout-Ou-Rien ; M1 : Milieu 1. (Schéma P. Herbepin, BiiGC)

Cette technologie a démontré son efficacité pour le maintien de la transparence de la cornée et améliore la survie des cellules endothéliales[28].

Comme souligné dans les principes de physiologie de la cornée (chapitre 1.1.2, page 11), l'environnement de l'endothélium et de l'épithélium cornéen sont différents. Dans le but d'optimiser la conservation de l'épithélium cornéen, nous avons tiré parti de la possibilité de séparer les compartiments épithéliaux et endothéliaux du bioréacteur. Cette séparation permet de maintenir le système mis en place pour la préservation du compartiment endothélio-stromal et de mettre en œuvre des conditions dédiées à l'épithélium cornéen. Ainsi, la chambre épithéliale est régulée de manière indépendante, et bénéficie d'un milieu de culture dédié et de la possibilité d'exposer l'épithélium cornéen de manière alternée à l'air ou au milieu de culture (**Figure 3**).

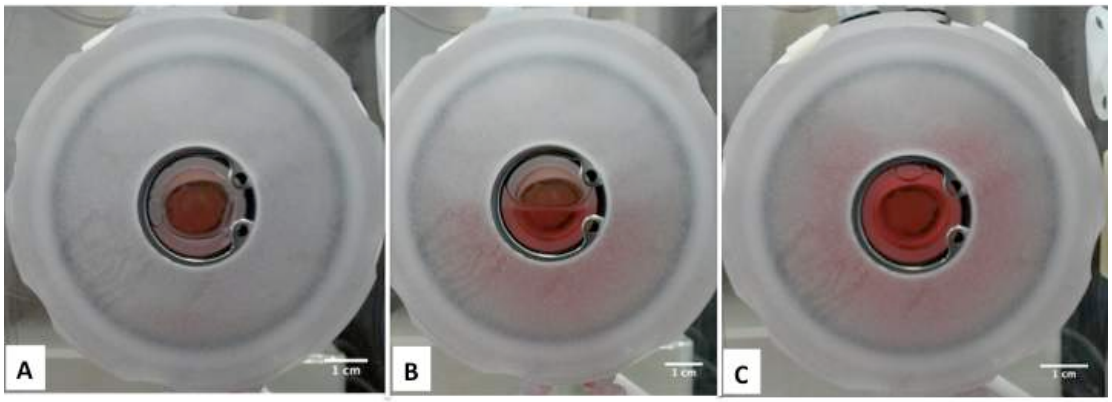



**Figure 3. Photographie du bioréacteur avec système alternant immersion/airlifting avec visualisation du compartiment épithélial.**

Ici, Il s'agit d'une version porcine du bioréacteur avec un capot comportant une vitre permettant une observation parfaite du compartiment épithélial et de la cornée. A : compartiment épithélial vide ; B : compartiment épithélial en cours de remplissage ou de vidange ; C : compartiment épithélial rempli. Barre : 1cm.

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Le bioréacteur a des parois transparentes pour permettre la réalisation de contrôles optiques tout en préservant la stérilité du greffon. L'accès facile au milieu de culture permet d'ajouter des molécules dans le compartiment désiré.

### 2.4. Autres systèmes *ex vivo* actifs

Nous dressons ci-après un panorama des modèles actifs de conservation *ex vivo* publiés (Tableau 1). Les principales critiques pouvant être formulées à propos de ces systèmes :

- Les cornées placées en chambre de perfusion ne peuvent être conservées que pour une durée limitée en raison de l'absence de confinement de la cornée dans un environnement hermétique et stérile.
- Les plateaux inclinés tournants (« rocking system ») sont encombrants, offrent une possibilité limitée de manipulation de la cornée, et n'assurent pas la préservation de l'endothélium cornéen, ce dernier étant placé au contact d'un support en agar.

- Les différents modèles développés ne permettent pas une manipulation facile des cornées et de réaliser facilement imagerie multimodale des cornées (OCT, microscopie spéculaire, etc.).

**Tableau 1. Revue de la littérature des systèmes actifs de conservation de cornées ex vivo.**

| Auteurs | Année | Espèce | Système | Conservation maximale | Conservation de l'épithélium | | Application | Évaluation de l'épithélium |
|---|---|---|---|---|---|---|---|---|
| | | | | | Milieu | Air | | |
| Brunette et al.[29] | 1989 | Lapin, Humain | CP | 3 sem. | DMEM | oui | Physiologie endothéliale | non étudié - rapporté comme endommagé |
| Richard et al.[30] | 1991 | Humain | PIT | 3 sem. | Milieu spécifique | oui | conservation | Histologie |
| Tanelian et al.[31] | 1992 | Lapin | CP | 70h | - | oui | cicatrisation | Coloration à la fluorescéine, Histologie |
| Thiel et al.[32] | 2001 | Porc | CP | 15h | BSS | oui | toxicité | Pénétration de la fluorescéine, Histologie |
| Zhao et al.[33] | 2006 | Bovin | CP | 10 j | MEM+4%FCS | oui | cicatrisation | Histologie, MEB, coloration à la fluorescéine |
| Piehl et al.[34] | 2010 | Porc | PIT | 21 j | Milieu spécifique | oui | cicatrisation | Coloration à la fluorescéine, Histologie |
| Schrage et al.[35] | 2012 | Lapin | CP | 3 j | Larmes artificielles | oui | cicatrisation | Coloration à la fluorescéine, Histologie |

| Auteurs | Année | Espèce | Système | Conservation maximale | Conservation de l'épithélium | | Application | Évaluation de l'épithélium |
|---|---|---|---|---|---|---|---|---|
| | | | | | Milieu | Air | | |
| Deshpande et al.[36] | 2015 | Lapin | PIT | 4 sem. | Milieu spécifique | oui | cicatrisation | Histologie, Immunohistochimie |
| Schmid et al.[37] | 2017 | Porc | CP | 7 j | Milieu spécifique | oui | conservation | Coloration à la fluorescéine, Histologie, RTE |

CP : chambre de perfusion ; PIT : plateau incliné tournant ; MEB : microscopie électronique à balayage ; sem. : semaines

# 3. Déroulement du projet de thèse

La première partie du projet a initialement visé :

- A développer le système permettant de réguler de manière indépendante la chambre épithéliale et permettre une exposition alternée à l'air et à un milieu de culture dédié aux cellules épithéliales
- A développer une version du bioréacteur adaptée à la conservation des cornées porcines

Cette première étape a été réalisée sur des cornées porcines en raison de la disponibilité et de l'accessibilité à ces dernières, permettant de réaliser le développement et la validation du système. De plus, les tissus sont disponibles après un délais post-mortem très court.

Cette première partie a fait l'objet d'une publication.

La seconde partie visait à adapter le système développé et évaluer son intérêt sur des cornées humaines provenant d'organoculture. Les cornées utilisées ont été récusées pour la greffe de cornée, en raison d'une densité en cellules endothéliales insuffisante principalement, ou de sérologie non conclusives. D'après le rapport 2012-2016 de l'Eye Bank Association of America, on estime à 25 to 39% la proportion de cornées prélevées par les banques de cornées mais qui ne pourront être utilisées pour les greffes de cornées[38], ce qui représente de 30 à 50.000 cornées par an aux États-Unis.

La valorisation de ces tissus apparaît comme essentielle. Ces cornées, d'origine humaine, peuvent avoir une grande valeur scientifique. Cependant, les cornées sont généralement qualifiées comme « non viable » pour la greffe après plusieurs jours de conservation. Des anomalies de l'épithélium cornéen sont déjà observable[21]. Pour valoriser ces cornées, nous avons testé la capacité du bioréacteur à réhabiliter l'épithélium cornéen de ces cornées « non viables », dans

l'espoir d'offrir dans le futur de nouveaux modèles expérimentaux utilisant des cornées d'origine humaine.

La publication de cette deuxième étude est en préparation.

# 4. Article 1 : conservation de cornées porcines en bioréacteur

Cet article a été publié dans la revue *Investigative Ophthalmology & Visual Science.*

Référence :

Storage of Porcine Cornea in an Innovative Bioreactor.

Guindolet D*, Crouzet E*, He Z, Herbepin P, Jumelle C, Perrache C, Dumollard JM, Forest F, Peoc'h M, Gain P, Gabison E, Thuret G.

Invest Ophthalmol Vis Sci. 2017 Nov 1;58(13):5907-5917. PMID: 29164231

# Storage of Porcine Cornea in an Innovative Bioreactor

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DG and EC contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: May 14, 2017
Accepted: October 19, 2017

Citation: Guindolet D, Crouzet E, He Z, et al. Storage of porcine cornea in an innovative bioreactor. *Invest Ophthalmol Vis Sci*. 2017;58:5907–5917. DOI:10.1167/iovs.17-22218

**Purpose.** To quantify cell survival and tissue structure preservation of porcine cornea stored in a bioreactor (BR) that recreates a transcorneal pressure gradient equivalent to intraocular pressure (IOP) and renews the medium.

**Methods.** A BR comprising endothelial and epithelial chambers was machined in a biocompatible material. The porcine cornea, securely held, separated the chambers. Medium flow and pressure inside the endothelial chamber were maintained by a peristaltic pump. In the epithelial chamber, the corneal surface was alternatively exposed to air and a specific medium. Two transparent windows allowed thickness measurement by optical coherence tomography without opening the BR. Porcine corneas stored in the BR-on (pressure 20 mm Hg, flow 5 μL/min, temperature 31°C) were compared with (1) BR-off (no pressure or flow); (2) organ culture; and (3) Petri dish with agar on the endothelial side. Epithelial and limbal structure and differentiation, corneal transparency and thickness, and endothelial viability were compared after 7 days of storage and with fresh corneas.

**Results.** Corneas stored in the BR-on were thinner and more transparent than those stored with the other methods. The BR-on preserved a stratified and differentiated (K3/K12+) corneal epithelium and undifferentiated basal limbal cells with stemness markers (K3/K12−; ABCB5, K14, p75+), as well as endothelial integrity.

**Conclusions.** By recreating equivalent IOP and medium renewal, the BR obtained unprecedented storage quality of porcine corneas and preserved their main epithelial, limbal, and endothelial characteristics.

Keywords: cornea, porcine, bioreactor, epithelium, storage

Ex vivo storage of the cornea in its physiological state is the holy grail of both eye bankers and researchers.

In eye banks, both storage methods currently used worldwide (cold storage in the United States, organ culture in Europe)[1] were introduced 40 years ago, mainly for their ability to ensure endothelial survival. They have rendered huge service, but corneas sustain irreversible alteration during storage, notably endothelial cell (EC) loss hundreds of times faster than during donor life.[2–4] The decrease in the endothelial reserve very likely influences graft survival. The survival of the epithelium is also altered,[5] but its minor role during corneal transplantation makes it a small concern for eye bankers. Stromal swelling during corneal storage is only partially reversed by osmotic molecules (e.g., dextran).[6] The overall increase in stromal thickness is likely to delay visual recovery (compared to fresh grafts), as complete deswelling takes at least 1 month.[7,8]

Fundamental applied and preclinical research in all fields involving corneal tissue (from ophthalmology, through cosmetics, to general drug testing) would also greatly benefit from a corneal storage method that maintains the integrity of the three corneal layers and their close interrelationship. Such a system could use corneas from food-industry animals that are normally discarded. These tissues could be collected and used immediately post mortem for preclinical tests, limiting the use of laboratory animals and providing an alternative assay in line with animal experimentation rules.[9] Animal cornea availability allows many experiments at reduced cost. Animal corneas are thus an unlimited research resource in theory, but in practice there is no satisfactory animal model of medium- to long-term corneal storage for easily available species: Ex vivo animal corneas, much more than human ones, quickly and dramatically swell and lose transparency after immersion in storage media. In the past, various irritancy assays were developed: excised corneas stored in a Petri dish using agar to fill the endothelial side of the cornea,[10–12] artificial anterior chambers such as those used for the Ex Vivo Eye Irritation Test (EVEIT)[13] or the bovine or porcine corneal opacity and permeability test.[14,15] None of these models extends corneal storage with concomitant epithelial and endothelial preservation, which is

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critical given the reported interactions between these two layers; epithelial cell differentiation[16] and epithelial basement membrane formation[17] are influenced by EC function.

Corneal physiology is complex and partly unexplained. Control of cornea shape, transparency, and thickness requires an endothelial pump; epithelial integrity, especially with mature watertight continuous tight junctions between the most superficial cells; and normal intraocular pressure (IOP). The biochemical composition of the stromal extracellular matrix explains its natural swelling when immersed in water. This swelling pressure (SP) (60 mm Hg in humans and pigs) is counterbalanced by the IOP (15 ± 5 mm Hg), which constantly pushes the posterior corneal surface forward, and by the constant water efflux exerted by the endothelial pump. This pump controls the imbibition pressure (IP), and the equilibrium can be summarized by the equation IP = IOP − SP.[18] After donor death, and even more so after corneal excision, IOP loss and the concomitant gradual loss of endothelial pumping function explain the rapid swelling of the cornea. Moreover, preservation of corneal epithelial cells and limbal epithelial stem cells requires a specific culture medium, intermittent air exposure,[19] and possibly shear stress.[20] Models with intact and functioning epithelial cells are needed to study normal and pathologic epithelial wound healing, and to develop a reliable eye irritancy assay.

To provide a more efficient storage method, we designed a corneal bioreactor (BR) that recreates the IOP in the endothelial compartment and the renewal of nutrient medium. A first version aimed to improve the condition of pregraft human corneas. A second version added intermittent epithelium exposure to air. In the present work, we assessed the integrity of the three corneal layers after 7 days' storage in this second version of our BR, which stores the cornea in a more physiological environment than other methods.

## Materials and Methods

### Corneal Bioreactor

The BR applies principles of previously described perfusion chambers and anterior artificial chambers,[12,21–26] but also has several innovations (Fig. 1). It was designed to maintain a sterile closed environment enabling long-term storage. The cornea is tightly secured to the BR base, using the scleral rim as a watertight seal to separate the epithelial and endothelial chambers; each chamber is connected to a distinct culture medium control system. A peristaltic pump controlled by a pressure sensor and a microcontroller continuously renews the culture medium (CM) at a rate of 5 µL/min, while creating a pressure 20 mm Hg higher than atmospheric pressure in the endothelial chamber. The CM was a minimum essential medium–based corneal organ CM containing 2% fetal calf serum (CorneaMax; Eurobio, Les Ulis, France). The epithelial chamber is connected to a peristaltic pump controlled by a microcontroller, alternating exposure of the epithelium to air and immersion in a CM (hereafter called air-lifting). A specific epithelial CM (hereafter called supplemental hormonal epithelial medium [SHEM]) consisted of Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA)/Ham's F12 (Sigma, Saint-Quentin-Fallavier, France) (2:1) supplemented with 10% fetal bovine serum (Eurobio), 10 ng/mL epidermal growth factor, 1% antibiotic antimycotic solution (Sigma), 0.1 nM cholera toxin (Sigma), 5 mg/mL insulin (Sigma), 5 µg/mL transferrin (Sigma), 0.18 nM Adenin (Sigma), 0.4 µg/mL hydrocortisone (Sigma), 1 µg/mL bovine pituitary extract (Thermo Fisher Scientific), and 2 nM 3,3′,5 Triiodo-L-thyronine (EMD Millipore, Burlington, MA, USA). The cornea was exposed to air and to SHEM for 30 and 1 seconds, respectively. The epithelial CM (25 mL) was changed every 2 days. In some of our experiments, the SHEM was replaced by CorneaMax. The BR with aforementioned operating endothelial and epithelial CM control systems is referred to as BR-on. Two transparent windows, on each side of the cornea, enabled optical control during experiments. The BR was designed to be compatible with current ophthalmology imaging systems, such as slit lamp, optical coherence tomography (OCT) systems, and specular microscopy. For the following experiment, prototype BRs were machined in a biocompatible material and included optical quality windows (0.9-mm-thick sapphire windows; France Fourniture Horlogerie, Vence, France). The complete system, except its control panels, was placed in a 31°C dry 5% $CO_2$ incubator.

Animals were used according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyeballs of 6-month-old pigs were obtained from a local slaughterhouse within 4 hours after death and transported to the laboratory on ice. Extraocular muscles and conjunctival tissue were removed. Eyeballs were decontaminated by two successive immersions for 5 minutes in 10% and 5% povidone–iodine solutions (Betadine; Meda, Merignac, France) and rinsed in balanced salt solution (Alcon, Rueil Malmaison, France) supplemented with 1/200 antibiotic antimycotic solution (Sigma). In five cases, central corneal thickness (CCT) was measured with OCT (see below) on the intact eyeball just before corneoscleral excision. The corneas were then excised under a laminar flow hood, in aseptic conditions, using forceps and a surgical scalpel blade, leaving a 3- to 5-mm scleral rim.

To check whether water could enter the ex vivo cornea via the lesions caused by iris root stripping and thus contribute to stromal swelling, the iris root was preserved in a subgroup of corneas. However, partial resection of the iris around the pupil was performed with scissors, as a preliminary experiment had shown harmful contact between the endothelium and the iris left intact.

Storage in the BR-on was compared with three other methods: (1) BR-off—corneas were placed in a BR base without CM control system (i.e., without pressure equivalent or continuous endothelial CM renewal, and without exposure to air of the epithelial chamber); endothelial (2 mL) and epithelial (10 mL) culture media were renewed every 2 days; (2) organ culture (OC), with corneas totally immersed in CM, as in eye banking; (3) agar system—corneas were mounted in a Petri dish on agar on the endothelial side and bathed with epithelial CM. The latter is not a corneal storage method, but has been validated for maintaining epithelial integrity.[7] Each method is detailed below and summarized in Table 1. All corneas were stored for 7 days, longer than ever previously reported for whole porcine corneas. A group of 10 corneas was also studied fresh to provide baseline data.

**Organ Culture.** Corneas were immersed in plastic vials (Gosselin, Hazebrouck, France) containing 25 mL CorneaMax and incubated at 31°C, in an atmosphere of 5% $CO_2$ and 90% relative humidity, as the plastics could be permeable to $CO_2$.

**Agar System.** After excision and complete iris removal, corneas were placed on a concave silicon support, epithelial side down. The endothelial side was filled with 2% agar (Sigma) in phosphate-buffered saline (PBS) at 35°C in sterile conditions. Once the agar solidified, the cornea was placed in a six-well plate (Corning Costar, Sigma), epithelial side up, immersed in epithelium CM, and incubated at 31°C, 5% $CO_2$ and 90% relative humidity.

**End-of-Storage Analysis.** After 7 days' storage, all corneas were assessed in parallel for integrity of the central corneal and limbal epithelium, stromal characteristics, and endothelial viability.

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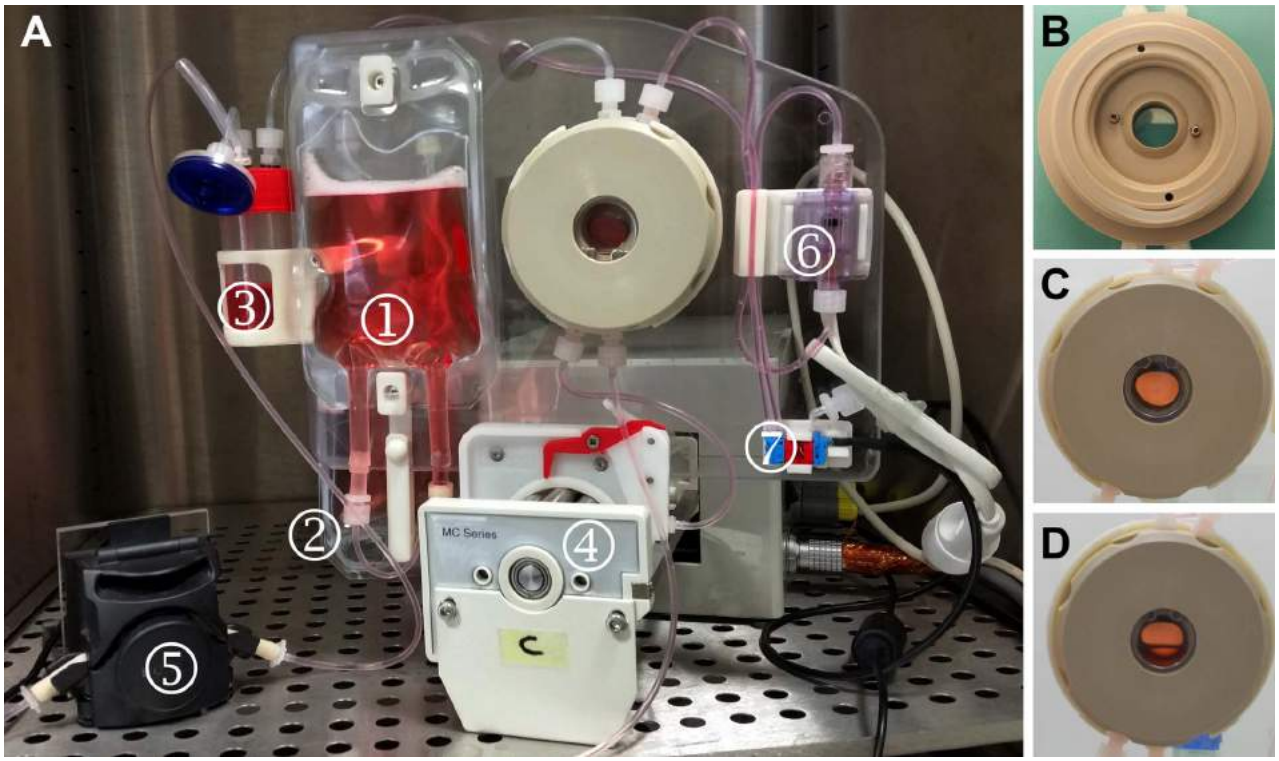



FIGURE 1. Main characteristics of the corneal bioreactor (BR) in a $CO_2$ incubator. (**A**) Overview of the BR in its cassette including the bag for fresh endothelial medium (*1*) and its waste (*2*), flask for epithelial medium (*3*), peristaltic pumps (*4*, *5*), pressure sensor (*6*), and micro solenoid valve (*7*). (**B**) Empty BR base with endothelial chamber and posterior window. (**C**) Porcine cornea in a BR after lid locking. (**D**) BR operating in corneal epithelium immersion phase.

*Epithelial Integrity.* The corneas were cut in two. One half was fixed in 4% paraformaldehyde for 24 hours at room temperature (RT), dehydrated through ascending concentrations of ethanol, and embedded in paraffin. Cross sections 7 µm thick were cut, rehydrated, and stained with hematoxylin, eosin, and saffron. Bright-field tagged image format file (TIFF) images of the cross sections were acquired using a microscope (IX81; Olympus, Tokyo, Japan). The other half of the cornea was embedded in optimal cutting compound (CellPath, Newtown, UK) and frozen using 2-methylbutane (Sigma) and liquid nitrogen. Samples were stored at −20°C. Tissue sections (14 µm thick) were cut using Cryostat Microm HM550 (Thermo Fisher Scientific) and spread out on Surgipath X-tra Adhesive (Leica Biosystems, Nussloch, Germany). After rehydration of tissue sections in distilled water for 15 minutes, nonspecific binding sites were blocked by incubation for 30 minutes at 37°C with blocking buffer, based on PBS supplemented with 2% heat-inactivated goat serum (Eurobio) and 2% bovine serum albumin (Thermo Fisher Scientific). Slides were incubated at 37°C for 1 hour with the primary antibody. Primary antibodies were supplied as listed in Table 2. Nonspecific rabbit and mouse immunoglobulin G (IgG; Zymed, Carlsbad, CA, USA) were used as primary antibodies for negative controls. These two controls were performed for each cornea. Secondary antibodies were Alexa Fluor 488 goat anti-mouse or anti-rabbit IgG or/and Alexa Fluor 555 goat anti-mouse or anti-rabbit IgG (Invitrogen, Eugene, OR, USA). Secondary antibodies diluted by 1:500 in blocking buffer were incubated for 1 hour at 37°C. Lastly, nuclei were counterstained with TO-PRO-3 Iodide (1/500) (Thermo Fisher Scientific) in blocking solution for 5 minutes at RT. Three rinses in PBS were performed between each step except between blocking of nonspecific protein

TABLE 1. Study Design

| Denomination | Iris | Epithelial Medium | Air-Lifting | Endothelial Medium | Endothelial CM Flow and IOP, mm Hg | *n* |
|---|---|---|---|---|---|---|
| BR-on with iris SHEM | With | SHEM | Yes | CMax | Yes/20 | 7 |
| BR-on without iris SHEM | Without | SHEM | Yes | CMax | Yes/20 | 5 |
| BR-off SHEM | With | SHEM | No | CMax | None | 4 |
| BR-off CorneaMax | With | CMax | No | CMax | None | 5 |
| BR-off CorneaMax for desiccation experiment | Without | CMax | No | CMax | None | 3 |
| Organ culture with iris | With | CMax | No | CMax | None | 6 |
| Organ culture without iris | Without | CMax | No | CMax | None | 6 |
| Agar system | Without | SHEM | No | None | None | 5 |

CMax, CorneaMax; *n*, number of replicates.

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Table 2. Primary Antibodies Used for Immunostaining

| Target Protein | Role (Expected Cell Compartment) | Animal Source | Dilution | Reference and Manufacturer |
|---|---|---|---|---|
| ABCB5 | Stemness (plasmic membranes) | Rabbit | 1/200 | NBP1-50547, Novus, Littleton, CO, USA |
| p75 | Stemness (plasmic membranes) | Rabbit | 1/200 | AB8874, Abcam, Cambridge, UK |
| K14 | Stemness (cytoplasmic) | Mouse | 1/200 | AB9220, Abcam |
| Laminin-5 | Basement membrane | Rabbit | 1/200 | AB14509, Abcam |
| K3/K12 | Differentiated corneal epithelial cells (cytoplasmic) | Mouse | 1/200 | AB68260, Abcam |
| ZO-1 | Tight junctions (apical plasmic membranes) | Rabbit | 1/200 | 40-2200, Invitrogen, Waltham, MA, USA |
| e-Cadherin | Epithelial cells (all plasmic membranes) | Mouse | 1/200 | 33-4000, Invitrogen |

binding sites and incubation with the primary antibody. Finally, the slides were mounted using Vectashield medium (Vector Laboratories, Burlingame, CA, USA). Images were captured with a confocal microscope (IX83 Fluoview FV-1000, Olympus), equipped with the Olympus Fluoview software.

*Stroma.* To analyze transparency, each cornea, in a Petri dish, was placed between a backlit chart and a camera.[27] Overall transparency underwent computerized analysis. Results are expressed as a percentage ranging from 0% to 100%, respectively, designating complete opacity and perfect transparency. CCT was assessed with an anterior segment optical coherence tomograph (OCT; CASIA SS-1000; Tomey, Tokyo, Japan). The cornea in the BR was placed in front of the OCT in a specially designed holder. Thickness was measured on the central cross section. The anterior and posterior limits of the cornea were automatically selected and manually corrected if necessary. The mean of five measurements performed on the central 3 mm was calculated.

*Pan-Endothelial Viability.* Viable endothelial cell density (vECD) was determined as previously reported.[28,29] Briefly, corneas were placed endothelial side up on a specific concave support to prevent corneal folds; iris root was stripped when present; then the concavity was filled with 150 μL Hoechst 33342 (8 μM; Sigma), calcein-AM (2 μM; Interchim, Montluçon, France), and ethidium homodimer (4 μM; Interchim) in PBS and incubated for 45 minutes at RT. Corneas were then gently rinsed in PBS. TIFF images were acquired using a fluorescent macro zoom microscope (MVX-10, Olympus), run by imaging software (CellSens Dimension, Olympus). A ×0.8 objective allowed observation of the whole porcine cornea in a single field. A z-stack of eight images spaced 600 μm apart, from the trabeculum to the endothelium center, was acquired with a filter for fluorescein isothiocyanate (FITC) fluorescence (ex: 450–490/em: 500–550 nm) for calcein and with a fluorescence filter for cyanine3 (CY3) (ex: 528/em: 617 nm) for ethidium. The in-focus images of the whole endothelium stained with calcein-AM and ethidium were reconstructed using the 3-D Extended Depth of Field (http://bigwww.epfl.ch/demo/edf, in the public domain) in ImageJ (National Institutes of Health, Bethesda, MD, USA). This image was used to calculate the area covered by viable, calcein-positive cells and with ethidium-positive cells. Images of Hoechst 33342–stained nuclei were acquired using a fluorescence filter for 4',6-diamidino-2-phenylindole (DAPI) (ex: 325–375/em: 450–490) with a ×10 objective on five nonoverlapping fields (a central image and one in each quarter). These images were used to determine the number of ECs per surface unit (mean of the five regions of interest). Image analysis was performed using the Cornea J plugin.[29] vECD was obtained by multiplying the average ECD calculated with Hoechst 33342–stained nuclei by the percentage of surface covered by viable ECs (calcein-positive).

## Dessication

To assess whether corneas remained hyperhydrated or had become hypertrophic or hyperplasic at the end of storage, three were dehydrated with air until they reached minimal thickness. Initially, they were stored in a BR-off for 7 days, fully immersed in CorneaMax. In these conditions, they reached maximum thickness (see Results) but the scleral rim, attached to the BR, could not retract. This mount also allowed easy OCT monitoring of corneal thickness and shape, contrary to OC, which precluded repeated measurements. At the end of storage, the pressure in the endothelial chamber was restored to 20 mm Hg. After the 30 minutes needed to reach a steady state, the lid was removed and the epithelium was left exposed to ambient air. The cornea was then dried with a continuous low flow of pressurized air until it reached a stable thickness on three consecutive measurements. CCT was assessed by OCT at the end of corneal storage in the BR-off, and then every 5 minutes.

## Statistical Analyses

Continuous nonnormally distributed variables were described by their median (10th–90th percentiles). The nonparametric Kruskall-Wallis test was used to compare quantitative variables between more than two independent groups, and the Mann-Whitney test for two independent groups. Post hoc analysis was performed using Dunn's multiple comparison tests to compare active BR and three other storage methods. The null hypothesis was rejected by a type I error $< 0.05$ ($\alpha < 0.05$). Statistical analyses were performed using SPSS 23.0 (SPSS, Inc., Chicago, IL, USA), and figures were produced using Excel (Microsoft, Redmond, WA, USA).

# Results

## Baseline Characteristics of Porcine Corneas

On postmortem excised corneas, epithelium status, transparency, CCT, and endothelial viability were considered the baseline criteria (Figs. 2–4). Central corneal epithelium was stratified with six to nine layers of differentiated cells expressing cytokeratin 3 and 12 (K3/K12) and E-cadherin+, lying on a basement membrane positive for laminin-5. The most superficial cells expressed zonula occuldens-1 (ZO-1) (Fig. 3). Limbal epithelium was stratified with six to nine layers of differentiated cells (K3/K12+) except basal limbal epithelial cells (K3/K12–), which expressed stem cell markers ABCB5+, K14+, and p75+ (Fig. 4).

## Bioreactor

Corneas stored in the BR-on without their iris were significantly thicker than corneas with the iris root left intact, with CCT of 2737 (1716; 3441) vs. 1253 (1035; 1559) μm, $P < 0.001$. The former had diffuse edema associated with massive peripheral edema in three of five cases, as shown in Figure 2. Maintaining corneal shape by the system of scleral mechanical locking prevented contact between iris remnants and the peripheral endothelium. Consequently, all corneas subsequently stored in

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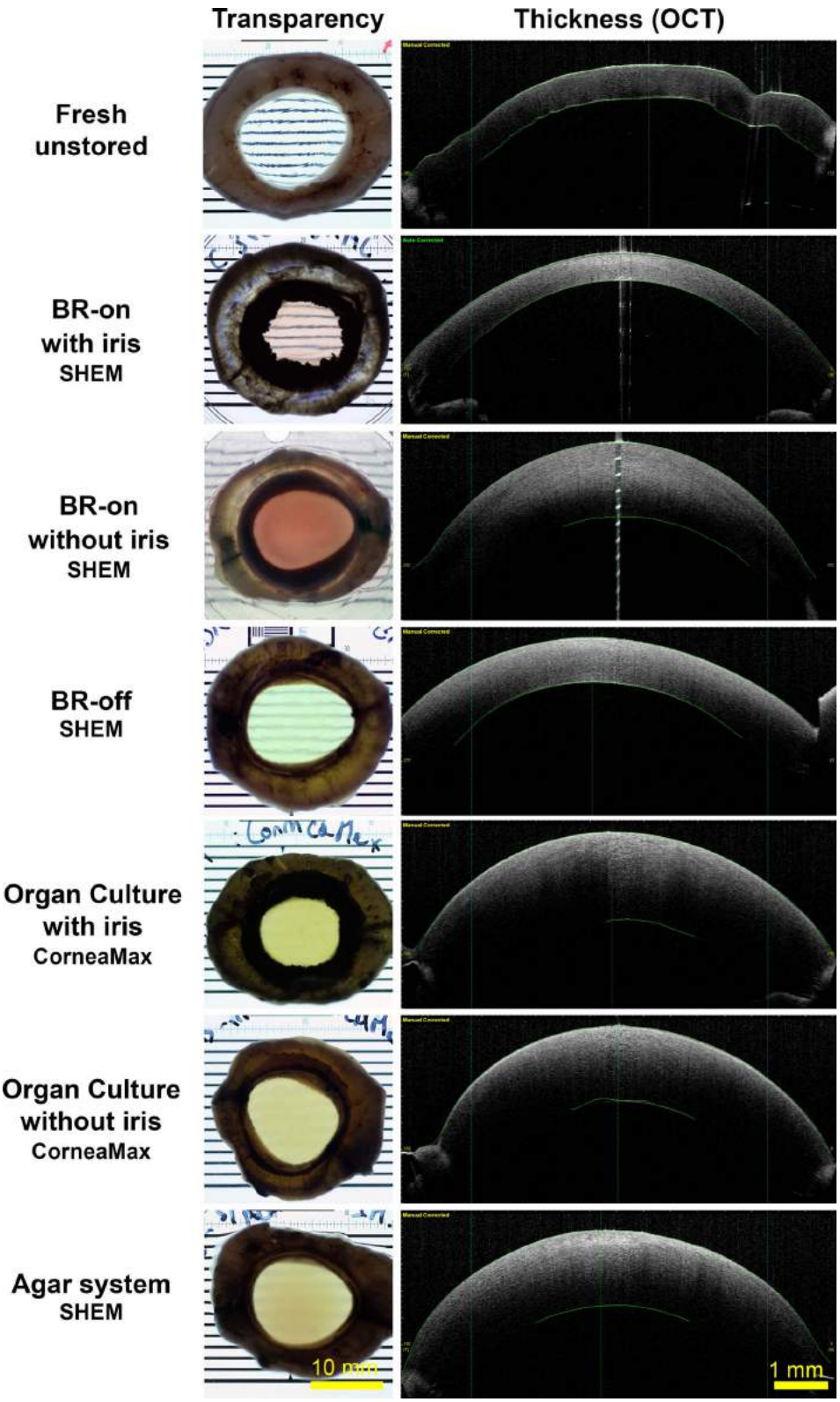



FIGURE 2. Transparency and central OCT scan of porcine corneas stored for 7 days in six different conditions. Fresh corneas were the control. Due to their natural lack of rigidity, they lost their dome shape. Except for corneas stored in the agar system, the endothelium was always in contact with a commercial organ culture medium (CorneaMax). For corneas in the BR and in the agar system, the epithelial side was immersed in supplemental hormonal epithelial medium (SHEM). For organ culture, corneas were simply immersed in CorneaMax. The BR-on was set with 20 mm Hg and 5 µL/min of medium renewal in the endothelial chamber, and the epithelium was alternatively exposed to air and to SHEM for 30 and 1 seconds, respectively.

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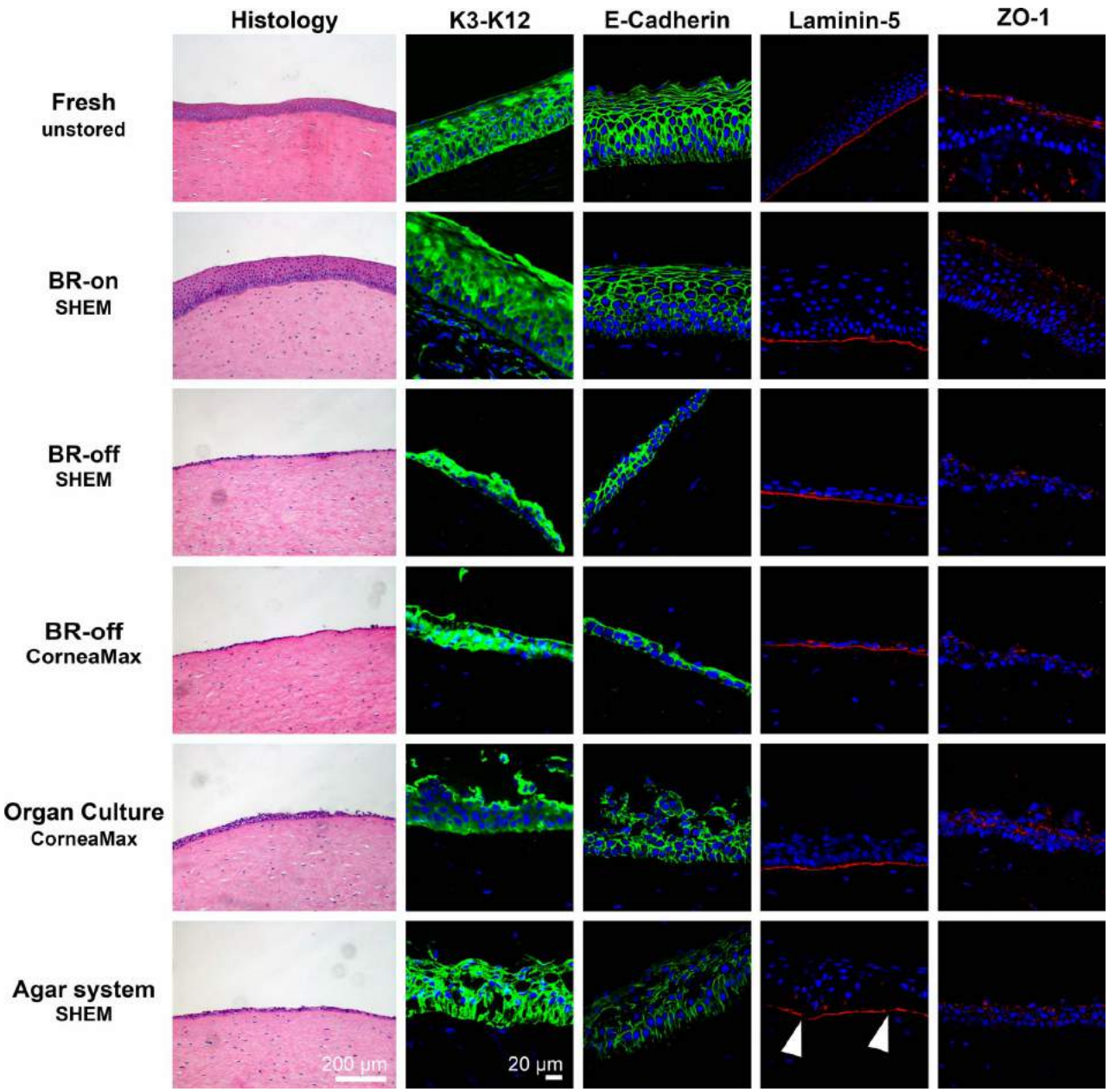



FIGURE 3. Histologic cross sections (hematoxylin, eosin, and saffron) and immunostaining of central epithelium of fresh porcine corneas (control) and after 7 days' storage in the bioreactor (BR)-on with 20 mm Hg and SHEM in the epithelial chamber, BR-off with either SHEM or CorneaMax in the epithelial chamber, organ culture in CorneaMax, and on agar with SHEM. Corneas in the BR had their iris root left intact. K3-K12 (*green*), E-cadherin (*green*), laminin-5 (*red*), ZO-1 (*red*). Nuclei were stained with TO-PRO-3 Iodide (*blue*). *Arrowheads* highlight irregularities in the basement membranes consistently observed only with the agar system. No staining was observed in negative controls (data not shown).

the BR and presented below had their iris root left intact. In these conditions, corneas stored in the BR-on had higher transparency and lower CCT compared to corneas stored using other methods (Fig. 5). Corneal and limbal epithelium organization was preserved; a stratified corneal (3 to 10 layers) and limbal (2 to 9 layers) epithelium was observed; limbal basal cells also had a small diameter and a high nuclear–cytoplasmic ratio. Immunohistochemical characteristics of the corneas stored in the BR-on were similar to those of postmortem corneas (i.e., a differentiated corneal epithelium lying on a regular basement membrane with tight junctions in the most superficial cells) (Fig. 3); limbal basal epithelial cells were undifferentiated and expressed stem cell markers (Figs. 3, 4). Mean vECD was 7% lower in the BR-on than in fresh corneas ($P = 0.088$), but 19% lower in the BR-off ($P = 0.001$). Corneas stored in the BR-off (with CorneaMax or SHEM in the epithelial chamber) had lower transparency and higher CCT (Figs. 2, 5) than corneas stored in the BR-on. Corneal epithelium stratification varied, ranging from no epithelium to an epithelium five cell layers thick when stored in CorneaMax and nine layers thick in SHEM (Fig. 3); limbal epithelium stratification ranged from one to six cell layers thick when stored in CorneaMax or SHEM (Fig. 4); stratification was slightly improved in corneas stored in SHEM in the epithelial chamber compared with CorneaMax.

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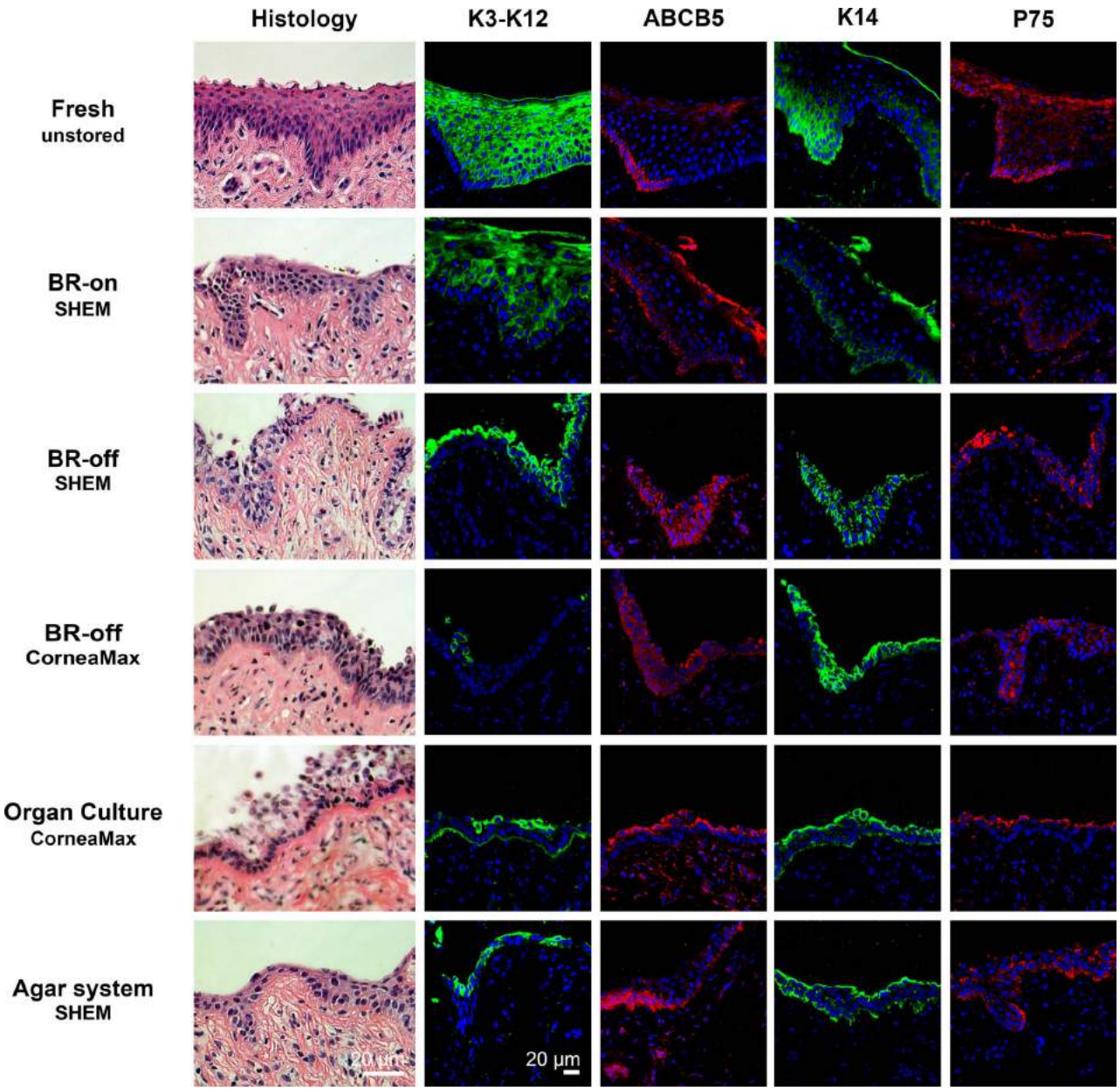



Figure 4. Histologic cross sections (hematoxylin, eosin, and saffron) and immunostaining of the limbus of fresh porcine corneas (control) and after 7 days' storage in the bioreactor (BR)-on with 20 mm Hg and SHEM in the epithelial chamber, BR-off with either SHEM or CorneaMax in the epithelial chamber, organ culture in CorneaMax, and on agar with SHEM. Corneas in the BR had their iris root left intact. K3-K12 (*green*), E-cadherin (*green*), laminin-5 (*red*). Nuclei were stained with TO-PRO-3 Iodide (*blue*). No staining was observed in negative controls (data not shown).

Corneal epithelium, when present, was differentiated (K3/K12+), with a preserved basement membrane (laminin-5); tight junction (ZO-1) expression was irregular (Fig. 3); clusters of basal limbal cells also preserved stem cell markers (Fig. 4).

## Organ Culture

Unlike what was observed with the BR-on, corneas stored with the iris root left intact were significantly thicker than iris-less corneas, with 3305 µm (2901–3726) vs. 2790 µm (2498–3137) $P < 0.001$. They also constantly presented dramatic EC mortality in their periphery, due to contact with iris remnants: A peripheral ring of approximately 2-mm width contained no ECs, and residual cells touching this zone were mostly stained with ethidium. As a result, all corneas subsequently stored in OC and presented below were iris-less. These corneas nevertheless lost all transparency and were dramatically swollen, some even losing their natural shape. Instead, they sometimes appeared as biconvex lenses with deep endothelial folds. Endothelial viability was reduced (Figs. 2, 5). The corneal epithelium was heterogeneous and mostly absent, except for a few areas of one to five layers of differentiated epithelium (K3/K12+; Fig. 3). A limbal epithelium one to five cell layers thick persisted, basal epithelial limbal cells were undifferentiated,

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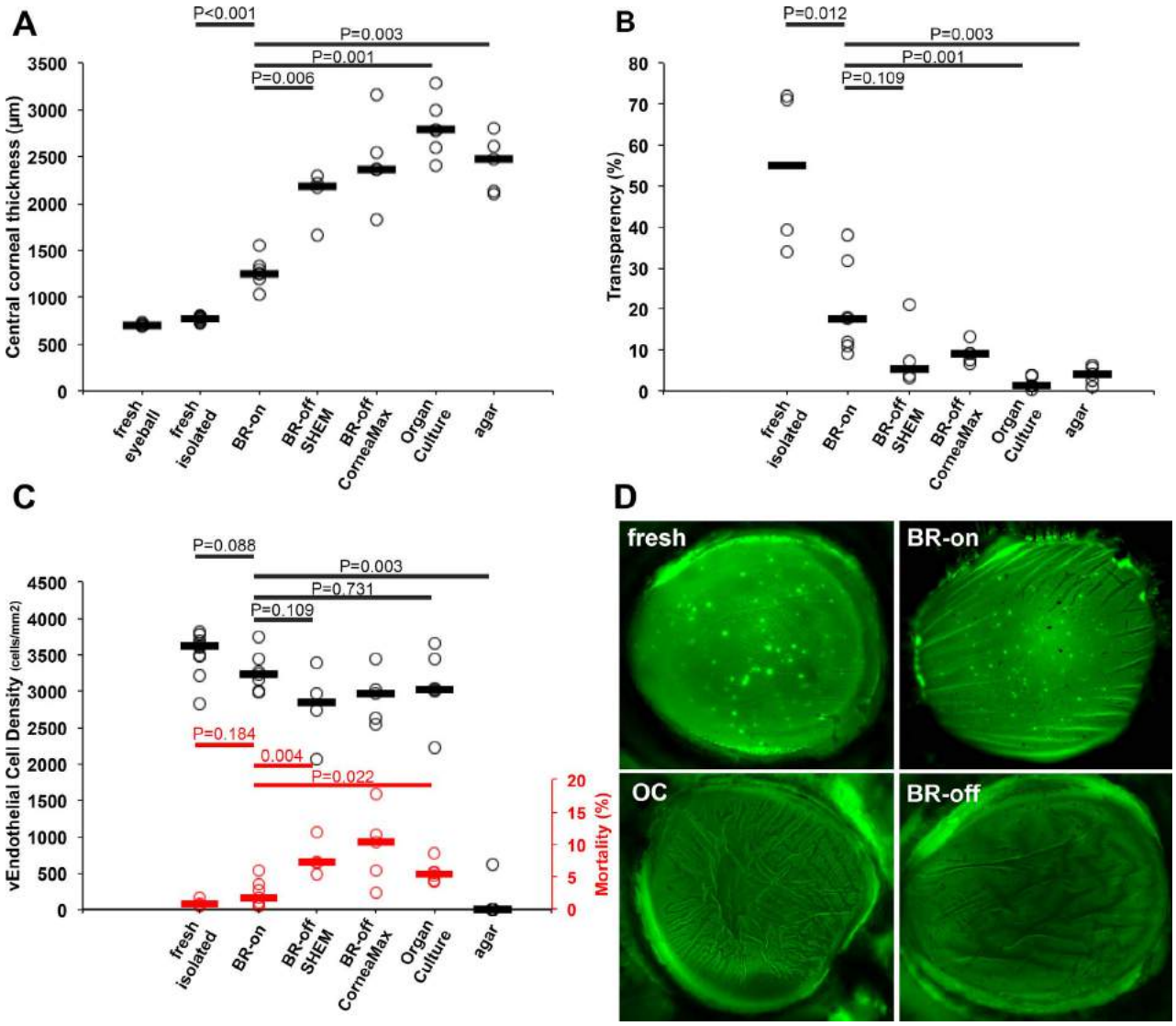



Figure 5. (A) Corneal thickness, (B) transparency, (C) viable endothelial cell density and % mortality of fresh porcine corneas and after 7 days' storage in five experimental conditions. CMax, commercial organ culture medium CorneaMax. Mortality was not quantified for the agar group because almost all cells had desquamed. (D) Endothelial viability determined by calcein-AM staining. In these four examples, the percentage of viable endothelium is 97% for the fresh cornea, 94% for the BR-on, 84% for OC, and 88% for the BR-off. In OC and in the BR-off, deep endothelial folds with stress lines are distributed on the whole cornea. In the BR-on, folds are absent in the center, and only mechanical folds appearing during cornea unlocking are visible in the periphery, with almost no cell mortality. Bright dots are artifacts, harmless for image analysis.

and cell clusters expressed stem cell markers (ABCB5, K14, and p75) (Fig. 4).

### Agar System

Corneas were also stored iris-less. These had reduced transparency and increased thickness (Figs. 2, 5). As expected, no ECs survived (except in one case, with extremely low vECD). The corneal epithelium was heterogeneous, and stratification ranged from one to four cell layers thick expressing K3/K12 (Fig. 3). A limbal epithelium two to five cell layers thick persisted; basal limbal epithelial cells were undifferentiated (K3/K12–), with clusters of cells labeled with stem cell markers ABCB5, K14, and p75+ (Fig. 4). Labeling of the basement membrane (laminin-5) was irregular and interrupted in a few areas; this feature was not observed with other storage methods (Fig. 3).

### Desiccation

Corneas were placed in the BR-off for 7 days, with a resulting CCT of 2281 (2274; 2284) µm ($n = 3$). Thirty minutes after restoration of the IOP equivalent, CCT was 2206 µm (2093; 2266). After approximately 120 minutes of exposure to dry pressurized air, CCT was 707 µm (617; 885) ($P = 0.02$ between end of storage and desiccation experiment). Corneal desiccation significantly and quickly reduced the CCT of corneas stored in the BR-off, indicating that increased CCT is linked to edema and not to a deposition of material in the stroma. Also, CCT of corneas stored in the BR-off tended to be lower than

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that of OC stored corneas, suggesting that scleral retraction may be involved in the observed stromal thickening of OC stored corneas.

## Discussion

Porcine corneas stored for 7 days in our BR-on with 20 mm Hg in the endothelial chamber, continuous renewal of the culture media, and epithelial air-lifting retain characteristics close to those of postmortem corneas for each of their three layers: epithelial, stromal, and endothelial.

Organ culture is clearly inappropriate for storing porcine corneas. The loss of IOP and of the epithelial barrier partly explains the dramatic stromal edema and subsequent EC loss in the present study. Corneas of pigs available from slaughterhouses are on average 6 months old, and are far less rigid than human corneas[30] after corneoscleral excision. Porcine cornea loses its shape, unlike human cornea. In OC, corneas of both species become edematous, but human cornea doubles in thickness and retains its shape, whereas porcine cornea becomes more than four times thicker and its shape is no longer recognizable. As SP is similar in both species,[31] the dramatic swelling capacity of porcine cornea may be due to a low level of natural collagen cross-linking compared with adult human corneas, for which age-related nonenzymatic cross-linking affecting the stromal collagen fibrils occurs.[32] In addition, corneal swelling starts immediately after scleral cutting (+70 μm in the present study), suggesting that the shrinking of naturally taut elastic collagen fibers plays a role in the increase in thickness.

The massive stromal edema observed in the BR-on only with iris-less corneas suggests that iris root stripping creates an abnormal entry route for water when IOP is restored; notably, this phenomenon was not observed in OC, as a pressure gradient was lacking. On the contrary, when the anatomy of the iridocorneal angle is preserved, the pressure restored in the BR partly offsets the SP and thus significantly limits stromal swelling; corneal swelling is also limited by the integrity of the epithelial barrier linked to tight junctions in the most superficial epithelial cells. Stored corneas thus remain thin without the addition of macromolecules, typically used by eye banks in conventional OC, to increase medium osmolarity. This result may have eye banking applications, as hyperosmolar deswelling media required during OC can be toxic to the endothelium.[33,34] In addition, reduction of corneal edema-related endothelial folds is likely to minimize EC loss, given the relationship between endothelial folds and EC apoptosis.[3,35] Further, continuous renewal of the CM in the BR could also improve EC survival. In the present study, vECD in the BR was not significantly higher than for OC, but the sample was small and storage time only 7 days. Using the same methods as in a clinical trial, the present data will be used to calculate prospectively the number of corneas to be included in a forthcoming experiment focused solely on endothelial survival, in order to compare endothelial survival in the BR and OC. Lastly, reduced stromal swelling in the BR was also logically associated with increased transparency. The BR has made possible ex vivo storage of a transparent porcine cornea for 7 days. This could be useful for ex vivo irritancy testing for longer than with the very short-term bovine or porcine corneal opacity and permeability tests.

Corneas stored in the BR-off tended to be slightly thinner than OC stored corneas. The only difference being that the scleral rim is tightly secured in the BR, we suggest that this limits the possibility of considerable tissue shrinkage observed in OC. Restoring the tautness of those scleral fibers that are interwoven with the corneal lamellae may help maintain corneal shape and thickness.

Corneas in the BR did not fully regain their physiological thickness. The possibility to obtain a further reduction of thickness by removing excess water through artificial drying of the cornea from the epithelial side reasonably consolidates the hypothesis of residual stromal edema and eliminates that of aberrant stromal hyperplasia. Four mutually nonexclusive hypotheses can be proposed to explain the residual edema: (1) abnormal passage of water via residual damage to the endothelium (not 100% viable); (2) hypofunctional endothelial pumps and/or an increase in intercellular permeability caused by imperfect composition of the OC medium (the culture media used in eye banks were developed more than 30 years ago, and should be revised to benefit from progress in cellular biology and EC physiology); (3) corneoscleral shrinkage begins immediately after trephination, before the rim is locked into the BR; (4) the 5% $CO_2$ atmosphere may favor anaerobic corneal metabolism directly involved in stromal edema.[36]

We implemented the second version of the BR, which included a system to alternately expose the epithelium to CM and air, thus recreating part of its physiological environment. The role of air exposure on the stratification of corneal epithelium, already demonstrated,[19] was also found in the BR. But no previous ex vivo model allowed both endothelium and epithelium to survive for 7 days. The specific BR environment, combining epithelium CM and intermittent air exposure, enabled preservation of a stratified and differentiated corneal epithelium and of limbal architecture with undifferentiated basal cells (K3/K12−) expressing stem cell markers (ABCB5+, K14+, p75+). Other authors developed rocking systems to alternate air and CM exposure on corneas in Petri dishes with agar, and obtained epithelial preservation for up to 4 weeks,[11,37,38] but the epithelium seemed less stratified and the integrity of the epithelial basement membrane and of the limbus was not studied. In addition, the air-lifting system of the epithelial chamber could induce a shear stress on the limbus that could be involved in epithelial stem cell regulation.[20] Preservation of the endothelium and epithelium is a prerequisite for developing a corneal wound-healing model that takes account of indirect interactions between these two layers described in the literature. Differentiation of epithelial cells is indeed influenced by the presence of endothelial cells.[39] Cases of limbal stem cell deficiency secondary to severe endothelial dysfunction were reported, which also supports the hypothesis of endothelium–epithelium interactions.[40] Moreover, basement membrane formation, one of the key regulators of epithelial wound healing and of epithelial–stromal interactions,[41] was associated in vitro with the presence of endothelial cells,[17] as demonstrated in a coculture model of epithelial cells in the presence or absence of endothelial cells. A drawback of the agar system is the absence of endothelium.[10,11,38] In our series, with this system, the basement membrane was irregular and also absent in some areas, whereas it is continuous and uninterrupted in other models with viable endothelial cells. Some irritancy assays use corneas mounted on an artificial anterior chamber[24,37,42] that preserves the endothelium, but these systems do not allow extended storage of corneas and are thus unsuitable for studying pathologic wound-healing processes. Tappeiner et al.[43] induced an epithelial wound in porcine corneas while preserving the whole eyeball, but only for 26 hours. The modified interactions between endothelium, stroma, and epithelium may explain the reduced stratification of epithelium preserved in the agar system and rocking systems described by Deshpande et al.[11] and Piehl et al.[12] compared to in vivo; moreover, no immunolabeling was done in these models to identify epithelial stem cell preservation. The present new

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model thus seems suited to studying normal and pathologic corneal epithelial wound healing and to develop an irritancy assay for screening new therapeutic molecules. In addition, the present study shows that the BR is an innovative experimental platform. It enables corneal storage in sterile conditions but also direct tissue visualization through two transparent windows without interrupting the experiment. Optical measurements such as OCT and transparency assessment can thus be performed in situ. The pressure inside the endothelial chamber can be adjusted if necessary, and the media bathing the endothelium and the epithelium can be different and have separately adjustable flows, as in the present study. In addition, media samples can be collected easily at various points in the circuit for specific analysis.

By restoring a near physiological environment to the cornea (IOP, flow, epithelial air-lifting, specific CM for each side), this BR version preserves porcine corneas for 1 week in conditions never previously achieved, with excellent EC survival, reduced thickness, shape maintenance, and integrity of the epithelium and limbus. Though still improvable, this model could be used for preclinical experiments. Research is under way to extend storage duration and to create a pathologic wound-healing assay.

## *Acknowledgments*

Supported by The National Agency for the Safety of Medicines and Health Products (L'Agence nationale de sécurité du médicament et des produits de santé or ANSM), appel à projet recherche 2012, BANCO project, the Fondation de l'Avenir pour le recherche médicale appliquée, AP-RM-16-008 project, and the French National Research Agency (ANR-13-PRTS-0001), Agence Régionale de Santé Ile de France (Année Recherche).

Disclosure: **D. Guindolet**, None; **E. Crouzet**, None; **Z. He**, None; **P. Herbepin**, None; **C. Jumelle**, None; **C. Perrache**, None; **J.M. Dumollard**, None; **F. Forest**, None; **M. Peoc'h**, None; **P. Gain**, Théa Laboratory (C), Quantel Medical Laboratory (C), P; **E. Gabison**, None; **G. Thuret**, Théa Laboratory (C), Quantel Medical Laboratory (C), P

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# 5. Article 2 : amélioration de la préservation et de la différenciation de l'épithélium de cornées humaines en bioréacteur

Le manuscript intitulé « Improved preservation and differentiation of the epithelium of human corneas stored in an active storage machine » est en cours de soumission à la revue *Investigative Ophthalmology & Visual Science.*

Le manuscrit est présenté en Annexes 1.

# 6. Conclusion

Le bioréacteur a permis de préserver les cornées porcines, notamment leur épithélium, dans un état proche de celui observé en *post-mortem* précoce. De manière plus marquante, l'architecture et la différentiation de l'épithélium de cornées humaines issues d'organoculture ont pu être améliorées, faisant entrevoir la possibilité d'utiliser ces cornées pour la réalisation d'expérimentations sur une plateforme spécialement adaptée.

Nos résultats suggèrent que la préservation de l'endothélium cornéen joue un rôle majeur dans la différenciation de l'épithélium cornéen et la préservation de sa membrane basale. En association avec un milieu de culture dédié à la culture épithéliale et une exposition intermittente à l'air (air-lifting), l'épithélium cornéen restore une différenciation proche de celle observée *in vivo*.

De manière surprenante, nous avons pu observer une augmentation du marqueur d'immaturité ABCB5 au niveau du limbe, suggérant une « activation » du limbe. La différenciation avancée de l'épithélium de la cornée centrale pourrait faire envisager une différenciation des cellules immatures limbiques en cellules d'amplification transitoire. Il serait intéressant d'étudier les marqueurs d'immaturité au niveau du limbe sur des cornées humaines conservées en bioréacteur de manière prolongée afin de confirmer qu'il s'agit de cellules d'amplification transitoire et de définit si un retour à un état d'homéostasie du limbe est possible, avec la préservation de cellules immatures basales.

Les applications possibles du bioréacteur en tant que plateforme expérimentale sont multiples : étude de la physiologie cornéenne, création de modèles de kératites infectieuses, greffe de cellules endothéliales,… Comme en témoigne une communication réalisée au congrès de l'European Association for Vision and Eye Research en 2018, offrant un aperçu des projets de recherche pouvant être effectués avec le bioréacteur (Annexe 2). Parmi les développements futurs, nous souhaitons créer un modèle de cicatrisation et de toxicologie sur cornées humaines conservées en bioréacteur. L'implémentation de tests fonctionnels, tel que l'évaluation la barrière épithéliale, fait partie des innovations en cours de développement. Le bioréacteur offrira

la possibilité d'étudier les interactions épithélio-stromales, en utilisant des cellules modifiées, qu'elles soient épithéliales ou stromales, mais également permettra d'étudier le remodelage stromale au cours de la cicatrisation.

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# Annexes 1: Improved preservation and differentiation of the epithelium of human corneas stored in an active storage machine

**Running head:** Surface of human corneas in an active storage machine

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Grants: Supported by The National Agency for the Safety of Medicines and Health Products (L'Agence nationale de sécurité du médicament et des produits de santé or ANSM), appel à projet recherche 2012, BANCO project, and by the Fondation de l'Avenir pour le recherche médicale appliquée, AP-RAM-16-008 project.

## ABSTRACT

Purpose. To characterize the corneal (CE) and limbal (LE) epithelium of human corneas stored in an innovative active storage machine (ASM).

Methods. Human corneas unsuitable for transplantation and previously stored at 31°C in a standard commercial organ culture (OC) medium for 2 to 6 weeks, were transferred into our ASM. The ASM allowed to actively maintain an overpressure on the endothelial side (20 mmHg) while ensuring the renewal of the OC medium (5 µL/min). We compared 3 modalities of storage in the epithelial chamber of the ASM: 1/alternating exposure to a supplemental hormonal epithelial medium (SHEM) and air (air-lifting); 2/continuous immersion in SHEM, 3/continuous immersion in standard OC medium. Passive immersion of the whole cornea in OC medium or in SHEM were used as control. Criteria after a 14-day storage: histology, ultrastructure by transmission electron microscopy and immunohistochemistry.

Results. The ASM with air-lifting was the most efficient to restore a pluristratified and differentiated CE (apical Zo-1 and MUC16 staining and restauration of the glycocalyx). In addition, the LE was stratified with preserved expression of ABCB5. The ASM with immersion in SHEM or OC medium restored a less stratified and differentiated CE. The passive storage in SHEM and OC led at best to a pauci-stratified CE with reduced differentiation.

Conclusions. The ASM enables to restore a corneal epithelium of human corneas stored *ex vivo* with differentiation characteristics close to normal. Rehabilitation of corneas discarded by eye banks will increase the availability of good quality tissue for research.


**Keywords:** cornea, human, machine perfusion, epithelium, storage

## INTRODUCTION

To answer fundamental or applied hypothesis and conduct preclinical research (e.g. drug testing) in the field of ocular surface diseases, ex vivo models using whole cornea have been developed to complete existing in vitro and in vivo models and to circumvent their respective drawbacks.

Ex vivo storage of corneas is based on the ability of the cornea, avascular, to survive outside the body when placed in an environment suitable for the preservation of the 3 main corneal cell types: epithelial cells, stromal keratocytes and endothelial cells. Beyond cell survival, the preservation of tissue integrity relies on the preservation of it structure and function. Depending on the scientific and medical questions asked, the complexity of the models is very variable and depends on the time of storage, and the use of animal or human tissues. The simplest models use fresh corneas preserved for only a few hours in buffer solutions. In more complex models that require extended storage, storage conditions are similar to long-term storage in eye banks using nutrient media derived from cell culture media. The use of fresh corneas from animals with histology and dimensions similar to the human cornea makes it possible to obtain acceptable models at a low cost compared to animal experimentation. However, readily available corneas from animals (bovine, pigs, rabbits and rodents) cannot be stored for more than a few days due to the development of major stromal edema. To address this problem, we recently reported that the restoration of intraocular pressure and control of media flows in a bioreactor, also called active storage machine (ASM), allowed fresh porcine corneas to be stored for 7 days, with preservation of epithelial integrity, endothelial viability and stroma hydration control that are unrivalled when compared to

conventional passive storage methods[1]. In this model the best epithelial structure was obtained by alternating exposure of the corneal epithelium to air and to a culture medium dedicated to corneal epithelium culture.

In addition, human corneas can also be used in ex vivo models to avoid physiological interspecies discrepancies and the need of specific antibodies against animal-specific epitopes. However, human corneas show post-mortem alterations of their epithelium quickly after death (desiccation, erosions)[2]. Corneal storage either at 4°C[3] or in organoculture (OC)[4] do not prevent these damages (sometimes complete abrasion, reduced stratification in most cases). Moreover, corneal donation is far from covering the needs for transplantation[5] and even less for research[6]. In Europe, fresh corneas used for research purpose, available immediately upon death, are by definition particularly rare because the donation must be intended for research. On the other hand, many corneas are disqualified for transplantation by eye banks. However, they have usually been stored for a short or long time in their storage medium and can no longer be considered as fresh corneas and may displays several modifications we previously listed.

Recently, we provided evidence that the ASM (with 20mmHg endothelial pressure and 5uL/min medium renewal) allows human corneas to be preserved for 4 weeks with much better endothelial survival, preservation of epithelial structure and differentiation and stromal thickness control than with passive OC conservation[7]. In this previous work, clinical grade human corneas were placed in the ASM immediately after corneal excision.

The purpose of the present work was to analyze the ASM's ability to regenerate the corneal epithelium of corneas disqualified by banks and already stored for several

weeks in OC. We compared several experimental conditions in order to optimize this regeneration so that rehabilitated corneas could be used for ex vivo experiments.

## MATERIAL AND METHODS

### Human corneas

The study was conducted in accordance with the ARVO statement for the use of human subjects in Ophthalmic and Vision research. Handling of donor tissues adhered to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision in protecting donor confidentiality. Human corneas unsuitable for transplantation were obtained from the eye banks of Saint-Etienne or Besancon after checking with relatives for the absence of opposition expressed during the lifetime of the subject, as authorized by French bioethics laws. Corneas were initially stored at 31°C in sealed glass flask of 100 mL OC medium containing 2% fetal calf serum (CorneaMax, Eurobio, Les Ulis, France) in a dry incubator, without medium renewal. Only corneas with between 2 and 6 weeks storage and an ECD of between 1000 and 2000 cells/mm$^2$ were included to match the corneas usually distributed by banks for research.

In order to obtain reference images of fresh human corneal epithelium in transmission electron microscopy (TEM), we analyzed the corneal button of a patient with a penetrating keratoplasty for keratoconus. In this disease, even if the epithelium is potentially involved in the pathophysiology[8], its architecture and histology are admitted as being very close to the healthy epithelium. In any case, it is one of the most easily accessible fresh controls. This corneal button was fixed within 10 minutes of trephination.

**Comparison of different storage conditions**

We compared the corneal and limbal epithelium after storage in ASM or in two static reference storage methods: conventional OC and a method used for epithelial wound healing assay using corneas mounted on an agar base (hereinafter referred to as agar system). The specificities of each of the techniques were detailed below and summarized in the Table 1.

***Active storage machine***

The ASM could be assimilated to the machine perfusions used for vascularized organs. We used the same model, machined in a biocompatible material (Polyether ether ketone), as we recently used for studying the long-term eye banking of human corneas [1,7]. Briefly, it maintained a sterile closed environment allowing long-term corneal storage and no-touch assessment through two optical quality windows in front of the epithelial and endothelial side or the tissue. Under a laminar flow hood, the cornea was tightly secured to the ASM base, using the scleral rim as a watertight seal to separate the epithelial and endothelial chambers, then the lid was closed on the epithelial side. In the version used in this work, a peristaltic pump controlled by a pressure sensor and a microcontroller continuously renewed the culture medium (CM) at a rate of 5 µL/min, while creating a pressure 20 mm Hg higher than atmospheric pressure in the endothelial chamber. The complete system, except its control panels, was placed at 31°C in a dry incubator with 5% $CO_2$, because some tubings of the ASM were $CO_2$ permeable and the buffer system of the media used was mainly a bicarbonate buffer requiring an atmosphere enriched to 5% $CO_2$. The ASM was used under 3 different conditions of increasing complexity: 1/ In

its simplest mode of operation (hereinafter referred to as ASM-Basic) with only one medium circulating first on the endothelial side and then on the epithelial side. This medium was a commercial organoculture medium based on DMEM and containing 2% fetal calf serum (CorneaMax). This mode of operation was the one previously used to validate storage in ASM for 4 weeks[7]; 2/ In the ASM-two-medium mode (ASM-2M), the endothelial chamber was filled with CorneaMax under the same circulation and pressure conditions as in ASM-Basic but the epithelial chamber was filled with a CM usually dedicated to in vitro epithelial cell culture (hereafter called supplemental hormonal epithelial medium, SHEM) that consisted of Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA)/Ham's F12 (Sigma, Saint-Quentin-Fallavier, France) (2:1) supplemented with 10% fetal bovine serum (Eurobio), 10 ng/mL epidermal growth factor (Sigma), 1% antibiotic antimycotic solution (Sigma), 0.1 nM cholera toxin (Sigma), 5 mg/mL insulin (Sigma), 5 µg/mL transferrin (Sigma), 0.18 nM Adenin (Sigma), 0.4 lg/mL hydrocortisone (Sigma), 1 µg/mL bovine pituitary extract (Thermo Fisher Scientific), and 2 nM 3,30,5 Triiodo-Lthyronine (EMD Millipore, Burlington, MA, USA). The SHEM was renewed at a rate of 5 µL/min using the same peristaltic pump; 3/ In the ASM-2M with air lifting mode (ASM-2M-AL), the previous mode was completed by a system alternating, in the epithelial chamber, exposure to the SHEM medium for 1 second and exposure to air for 30 seconds, thanks to a peristaltic pump and a microcontroller independent of the main ASM system. The SHEM (25 mL) was changed every 2 days.

***Organ Culture***

Corneas were immersed in glass vials containing 100 mL CorneaMax and incubated at 31°C.

***Agar System***

Corneas were placed on a concave silicon support, epithelial side down. The endothelial side was filled with 2% agar (Sigma) in phosphate-buffered saline (PBS) at 35°C in sterile conditions. Once the agar solidified, the cornea was placed in a six-well plate (Corning Costar), epithelial side up, immersed in SHEM, and incubated at 31°C, 5% $CO_2$ and 90% relative humidity.

**Table 1. Characteristics of the 5 corneal conservation methods compared in this work.** The active storage machine was used under 3 different conditions of increasing complexity and compared to 2 reference methods: organ culture and agar system.

| Denomination | Epithelial CM | Air-Lifting | Endothelial CM | Endothelial CM Flow and IOP, mm Hg | n |
|---|---|---|---|---|---|
| ASM-Basic | CMax | No | CMax | Yes/20 | 6 |
| ASM-2M | SHEM | No | CMax | Yes/20 | 3 |
| ASM-2M-AL | SHEM | Yes | CMax | Yes/20 | 12 |
| OC | CMax | No | CMax | No | 6 |
| Agar | SHEM | No | None | No | 3 |

ASM, active storage machine; B, Basic; 2M, two medium; AL, air-lifting; CM, culture medium; CMax, corneamax; n, number of replicates; OC, organ culture; SHEM, supplemental hormonal epithelial medium.

***Organization and aims of the comparisons***

In our previous study using the ASM, the storage period was extended to 4 weeks to match the long-term storage period in European eye banks. Here, the aim was to rehabilitate the epithelium disqualified corneas to make them quickly available for research. Preliminary analysis after 7 days of storage displayed results closed to the ones reported in this study. Nevertheless, the stratification was improved after extending the storage to 14 days. This compromise seemed sufficient to allow complete regeneration in the case of severely altered epithelium without excessively extending storage time for corneas already in OC for few weeks. In order to increase the power of the comparisons by minimizing the inter-individual variability related to the donor (age, genetics, pathology) and the storage prior to the experiments (time to death, OC duration), we used only pairs of corneas. Thus, each cornea of the pair was preserved with a different method. The comparisons were organized as follows, each time in triplicate: 1/ ASM-Basic versus OC to confirm the specific effect of active conservation after 14 days (the previous tests were performed over 28 days and with fresh corneas[7]); 2/ ASM-Basic versus ASM-2M-AL to analyze if air lifting with an epithelium-specific medium further improved its regeneration and differentiation; 3/ASM-2M-AL versus ASM-2M to characterize the specific contribution of air lifting; 4/ ASM-2M-AL versus OC and ASM-2M-AL versus Agar to compare the most sophisticated method with the 2 conventional passive methods.

**End-of-Storage Analysis**

After a 14-days storage, corneas were cut in three pieces and processed as follows.

***Histology.*** One fourth of the cornea was fixed in 4% paraformaldehyde for 24 hours at room temperature (RT), dehydrated through ascending concentrations of ethanol, and embedded in paraffin. Cross sections 7µm thick were cut, rehydrated, and stained with hematoxylin, eosin, and saffron. Bright-field tagged image format file (TIFF) images of the cross sections were acquired using a IX81 microscope (Olympus, Tokyo, Japan).

***Immunohistochemistry.*** One fourth of the cornea was immediately processed for flat-mounted immunostaining, as previously reported[7,9]. The sample was divided in two, one piece was fixed in 0,5% paraformaldehyde for 1 hour at room temperature (RT) and then permeabilized in 1% Triton (Sigma-Aldrich) for 10 minutes at RT; the sample was rinsed twice in PBS for 5 minutes between each step; the other piece was fixed in methanol for 1h at RT and then rehydrated in three bath of PBS for 5 minutes at RT. Nonspecific binding sites were blocked by incubation for 30 minutes at 37°C with blocking buffer, based on PBS supplemented with 2% heat-inactivated goat serum (Eurobio) and 2% bovine serum albumin (Thermo Fisher Scientific). Slides were incubated at 37°C for 1 hour or overnight at 4°C with the primary antibody diluted by 1:200 in blocking buffer. Primary antibodies were supplied as listed in Table 2. Samples were incubated for 2 hours at 37°C with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, Eugene, OR, USA) diluted by 1:500 in blocking buffer. Nuclei were counterstained with TO-PRO-3 Iodide (1:500) (Thermo Fisher Scientific) in blocking solution for 5 minutes at RT. Three rinses in PBS were performed between each step except between blocking of nonspecific protein binding sites and incubation with the

primary antibody. Finally, the slides were mounted using Vectashield medium (Vector Laboratories, Burlingame, CA, USA).

One fourth of the cornea was embedded in optimal cutting compound (Thermo Scientific) and snap frozen using pre-chilled 2-methylbutane (Sigma) and liquid nitrogen. Samples were subsequently stored at -80°C until further processing. Tissue sections (14µm thick) were cut using Cryostat Microm HM550 (Thermo Fisher Scientific) and spread out on Surgipath X-tra Adhesive (Leica Biosystems, Nussloch, Germany). Immunolabelling on cornea sections was done as previously described[1]: nonspecific rabbit and mouse immunoglobulin G (IgG; Zymed, Carlsbad, CA, USA) were used as primary antibodies for negative controls; incubation with the secondary antibody was reduced to 1 hour.

Images were captured with a confocal microscope (IX83 Fluoview FV-1000, Olympus), equipped with the Olympus Fluoview software. The image acquisition parameters (laser power, 10%) were maintained identical for both corneas from the same donor. For Zo-1 and MUC16 staining, maximum intensity projection was obtained from a Z stack of images using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/).

***Transmission Electron Microscopy.*** The corneal epithelium was examined by TEM as previously described[10]. One fourth of the cornea was fixed in 1% glutaraldehyde (16220; Electron microscopy sciences) and 0.5% paraformaldehyde (P/0840/53; Fisher Scientific, Loughborough, UK) in 0.02M $Na_2HPO_4$ (Prolabo, Paris, France)/0.08M $KH_2PO_4$ (P3786; Sigma) buffer (pH 7.4), postfixed in 0.1 M cacodylate-buffered 1% osmium tetroxide (19180; Electron Microscopy Sciences) for 1 hour, dehydrated in a graded series of ethanol, and embedded in Epon resin (Oxford Instruments, Oxford, UK). Ultrathin

sections of 90 nm were cut with an ultramicrotome (MTXL) and stained with 7% uranyl acetate (73943; Sigma) in methanol and Lead(II) citrate tribasic trihydrate (C6522; Sigma). Pictures were taken with a TEM (H-800; Hitachi) equipped with a charge-coupled device camera (XR41; AMT, Danvers, MA, USA). Three corneas from each storage condition were processed. Pictures were assembled using Adobe Photoshop CS6.

## RESULTS

The 30 corneas were obtained from 15 donors aged 76±37 (mean ±standard deviation, SD) years (56 to 91, median 78). Death to storage time and storage time were 13±7 hours (4 to 24, median 14) and 21±7 days (13 to 34, median 18), respectively. Endothelial cell density measured between day 2 to 5 by the eye bank was 1645±249 cells/mm$^2$ (1122 to 1992, median 1670).

### Corneal Epithelium (CE).

The CE of corneas stored in the ASM-Basic was more stratified compared to paired corneas stored in OC (**Figure 4**,**Figure 5**), confirming the beneficial effect of the ASM alone (IOP restoration, medium renewal). The CE organization was limited to 1 to 2 layers in OC whereas it was 3 to 4 layer-thick in the ASM-Basic. The CE of corneas stored in ASM-2M-AL was more stratified as compared to paired corneas stored in ASM-Basic or ASM-2M, with 5-7 layers in ASM-2M-AL, 3 to 4 layers in ASM-Basic (different triplicate from the line above) and 3-5 layers in ASM-2M, suggesting the additional role of the SHEM, specifically formulated for epithelial cells maintenance and the effect of intermittent air-lifting, respectively. Lastly, increased stratification was observed while

comparing corneas stored in ASM-2M-AL and paired corneas stored in static condition, i.e. either OC or on agar, that were limited to 1 to 2 layer-thickness.

Besides the reduced stratification in agar and OC groups, CE architecture was abnormal with enlarged basal cells (**Figure 4**). In the ASM (all conditions), the basal cells of the CE were columnar-shaped, superficial cells were flattened (squamous) and in-between polyhedral-shaped cells (wing cells) could be observed. Intercellular spaces could be observed between the most superficial cells and the cells located underneath in OC and agar groups, when multilayered. Additionally, a reduced number of desmosomes was observed in these 2 static storage conditions in comparison to corneas stored in the ASM. At the apical surface of superficial cells, microvilli could be observed in every storage condition. Nevertheless, their density was reduced in corneas stored in OC and agar as compared to corneas stored in the ASM (**Figure 5**). The extrinsic cell surface coat prominent on the tips of the microvilli, the glycocalyx, was observed in corneas stored in ASM-2M-AL (2 out of 3), ASM-2M (2 out of 3) and ASM-Basic (1 out of 3) whereas it was not observed in any cornea stored in static conditions (OC or agar). Vacuoles were observed mainly in basal cells of corneas stored in the ASM (all conditions) (**Figure 5**). To a lesser extent, theses vacuoles were also observed in the fresh cornea analyzed by TEM.

Whatever the storage condition, the CE expressed corneal differentiation markers (E-cadherin and K3/K12+; **Figure 4**) and relied on a continuous basement membrane (laminin-5; **Figure 4**). The most superficial layer of the CE displayed a ZO-1 staining (**Figure 4**). Nevertheless, the ZO-1 staining was irregular and interrupted in corneas stored in static condition, OC and agar, and paired corneas stored in ASM-Basic or ASM-

2M-AL displayed enhanced ZO-1 staining, showing the role of the ASM for CE differentiation. Corneas stored in ASM-2M-AL had also an enhanced ZO-1 staining in comparison to paired corneas stored in ASM-2M or ASM-Basic, suggesting the additional role of the culture media and of intermittent air exposure in corneal epithelial cell differentiation.

MUC16 staining was irregular in corneas stored in static condition (OC and agar (except for 1)); conversely it was more evenly distributed when cornea were stored in the ASM-Basic (5 out of 6) or ASM with SHEM with or without airlifting, (**Figure 4**).

**Limbal Epithelium (LE).**

The LE was also more stratified in corneas stored in the ASM as compared to paired corneas stored OC or agar (**Figure 6**). Similarly to what was observed in the CE, the LE was more stratified when stored in ASM-2M-AL in comparison to LE of corneas stored either in ASM-Basic or ASM-2M. ABCB5 staining was observed in every storage condition (**Figure 6**) and was not limited to the basal layer but also extend to suprabasal layers.

## DISCUSSION

After having shown that the ASM enables to keep fresh porcine[1] and human[7] corneas in conditions unparalleled until now by conventional storage methods, we show in this paper that the ASM promotes the epithelial regeneration of human corneas that were previously stored for a prolonged period in an eye bank. Corneas stored in the ASM that combines restoration of an IOP equivalent with continuous renewal of the CM in the

endothelial chamber, in conjunction with intermittent airlifting and exposure to a CM dedicated to epithelial cells in the epithelial chamber enabled to restore a more stratified and differentiated corneal epithelium in comparison to other storage conditions. The systematic use of pairs of corneas strengthens the value of our observations.

At least 3 factors may explain why static (or passive, i. e. without physiological pressure restoration) storage methods cannot preserve the integrity of the epithelium of animal[11] or human corneas[12–14] and why the ASM allows it: 1/The storage media, initially developed to preserve endothelial cells deprived of proliferative capacity, may be suboptimal for epithelial cells. Nevertheless, the formulation of the media does not seem to be the most important factor since we have observed the same epithelial alterations despite the use of a SHEM medium used in in vitro culture of human corneal epithelium; 2/ Its static nature. The only difference between the OC and the ASM-Basic is the restoration of IOP and the renewal of the CM, which is the same in the 2 experimental groups. This confirms that a dynamic storage with enhanced endothelial viability/function and better control of stromal hydration is involved in CE stratification; 3/ The absence of shear stress. Shear force[15] or air exposure[16] on the ocular surface contribute to corneal epithelium preservation and differentiation. In vitro, shear stress was associated to stemness maintenance of limbal epithelial stem cells[17], in maturation of superficial epithelial cells (flattened shaped, enhanced expression of tight junction protein)[15] and also in superficial cell shedding[18]. Corneal epithelium exposure to air is also a factor involved in its stratification and differentiation *in vitro*[16,19] and *ex vivo*[1,11]. Deshpande et al.[11] used a rocking system on rabbit cornea stored in Petri dish to reproduce intermittent moisture and air exposure (airlifting) of the corneal surface and showed an improvement

of corneal epithelium preservation. The ASM, initially developed for eye banking, was not focused on the survival of the corneal epithelium, as in penetrating keratoplasty or anterior lamellar keratoplasty, the donor epithelium is completely replaced by the one of the recipient[20]. Nevertheless, in this first version (ASM-Basic) the CM flow in the epithelial chamber may generate a shear stress at the surface of the most superficial epithelial cells but probably less efficient than air lifting. Airlifting was therefore implemented to a specific version (ASM-AL) used in this paper to enhance corneal epithelium maintenance.

Other evidence in the literature suggests the role of the endothelium in corneal and limbal epithelium health. In vitro experiments reported that coculture of endothelial cells with EC play a role in EC differentiation and epithelial basement membrane formation[21]. In clinical practice, the corneal stratification is impaired in bullous keratopathy and could be associated with limbal stem cell deficiency[22]. Altogether, these results suggest the interest of corneal endothelial cell preservation when developing an ex vivo model intended to study the corneal epithelium.

To favor CE preservation while reproducing ex vivo corneal physiological environment, perfusion chamber were developed for animal corneas (rabbit[23], porcine[1,24] and bovine[25]). The 2 advantages of animal corneas are their unlimited availability and the possibility of using them fresh, in the first few hours after the animal's death. On the other hand, extrapolation to humans may be limited. Brunette et al. developed a perfusion chamber for human corneas but their study was limited to the corneal endothelium[26]. Between 2012 and 2016, corneas not released for transplantation ranged from 25.2 to 38.7% of tissues harvested by eye banks according to the Eye Bank Association of America[27]. These corneas are invaluable for research, but the constant degradation of

quality, particularly of the epithelium, observed whatever the storage method, may limit some research[3,4]. In the ASM, the CE displayed improved stratification and differentiation characteristics in comparison to corneas stored in static conditions. Especially, we noticed enhanced ZO-1 staining that belong to the apical tight junction complex as well as glycocalyx restoration. These structures are observed in differentiated squamous corneal epithelial cells and are involved in the ocular surface barrier function (OSBF)[28]. OSBF is of paramount importance as its dysfunction is involved in the pathogenesis of several ocular diseases[28]. Therefore, restoration of OSBF in human cornea *ex vivo* could be useful to develop ocular surface disease models. Additionally, the bioavailability of topical drugs is influenced by the permeability barriers[29] such as apical epithelial glycocalyx[30]. Further developments are ongoing to implement functional test[28] able to test the OSBF because structural component of the OSBF may not necessarily correlate with the actual function. Additionally, we noticed a MUC16 staining in every storage condition, to different extents. While taking into account the apical structures (glycocalyx) that were observed in TEM, this result suggests that MUC16 expression cannot be considered by itself as a marker of OSBF restauration, highlighting the need for functional testing of the OSBF.

In humans, the corneal epithelium is renewed by cells migrating from the corneal limbus[31] that is the putative niches for LESC. For corneal epithelium homeostasis, LESC generates amplifying cells that proliferate, migrate and differentiate according to the X, Y, Z hypothesis[32]. The lack of LESC impairs CE renewal and leads to an invasion of the corneal surface by conjunctival epithelial cells (conjunctivalization)[33]. Numerous markers specific of the LESC were identified in an effort to characterize and isolate the most undifferentiated cells[34]. In homeostasis, these markers are usually expressed by the most

basal limbal cells. Nevertheless, the expression of these markers may not be restricted to the basal cells when the corneal epithelium is wounded[35]. In our study, the staining for ABCB5 in the limbus was not limited to the most basal layer conversely to what was previously reported previously[36]. This marker was identified as LESC markers[35,36] and also committed progenitor cells[37]. We hypothesize that the observed phenotype is related to an “activated limbus” in the context of the CE regeneration, similarly to what was reported for p63[35]. The observed phenotype could be associated to a healing phase and it would be interesting to assess ABCB5 localization after an extended period of storage. Additionally, a controversy still exist regarding role of limbal cells[38,39] in corneal epithelium homeostasis. Therefore, the role of the preservation of limbal should be further assessed.

This study has some limitations: 1/ we did not assess the rehabilitation at different time points (shorter or long-term). Indeed, it would be useful to know the minimum duration allowing the regeneration of a normal epithelium to further optimize the process, as well as the long-term survival of the newly formed epithelium. This first work enabled to select an effective method. In the future, we will determine the minimum time required for epithelial regeneration and whether it is possible to maintain the normal epithelial structure for several months; 2/ air-lifting can be optimized. No previous studies determined if an ideal time of air exposure could be advised and further work should be done for that purpose. In human the blinking rate is more frequent than the frequency we used. Nevertheless, the closed environment of the ASM should offer an increased humidity in the anterior chamber and limit desiccation; 3/ we will have to test the interest of ASM for corneas resulting from storage at 4°C, which are the most numerous in the world[5] in which the epithelium is also frequently altered[3,27]; 4/ We did not assess

functionally the epithelial barrier function that is subject of ongoing development in the laboratory.

## CONCLUSION

The corneal epithelium stratification and differentiation of human corneas non suitable for corneal transplant can be enhanced after storage in an ASM that restore near physiological environment to the cornea (IOP, medium flow, epithelial air-lifting, specific CM for each side). The possibility to regenerate the epithelium from corneas disqualified for clinical use by eye banks could be a new source of tissues for research in the ocular surface field.

**Legends.**

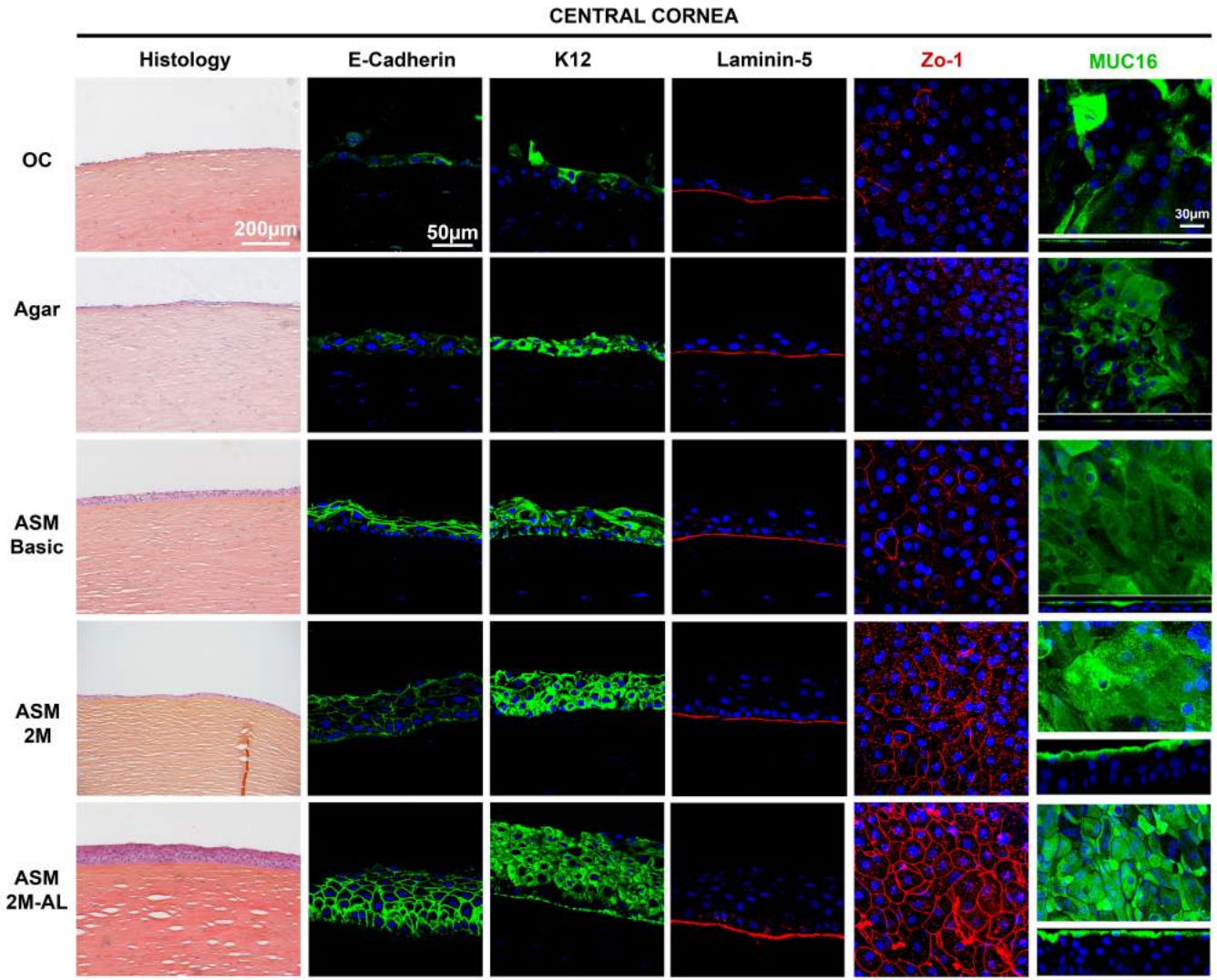

CENTRAL CORNEA
Histology
E-Cadherin
K12
Laminin-5
Zo-1
MUC16
OC
Agar
ASM Basic
ASM 2M
ASM 2M-AL
200µm
50µm
30µm

**Figure 4. Histologic cross sections (hematoxylin, eosin, and saffron) and immunostaining on sections or on flat-mounted of the corneal epithelium of human corneas after 14 days' storage in organ culture, on agar or in the active storage machine (ASM). K3-K12 (green), E-cadherin (green), laminin-5 (red), ZO-1 (red) and MUC16 (green). Nuclei were stained with TO-PRO-3 Iodide (blue). MUC16 staining pictures presented are the en face (upper image) and the orthogonal (bottom image) views. No staining was observed in negative controls (data not shown).**

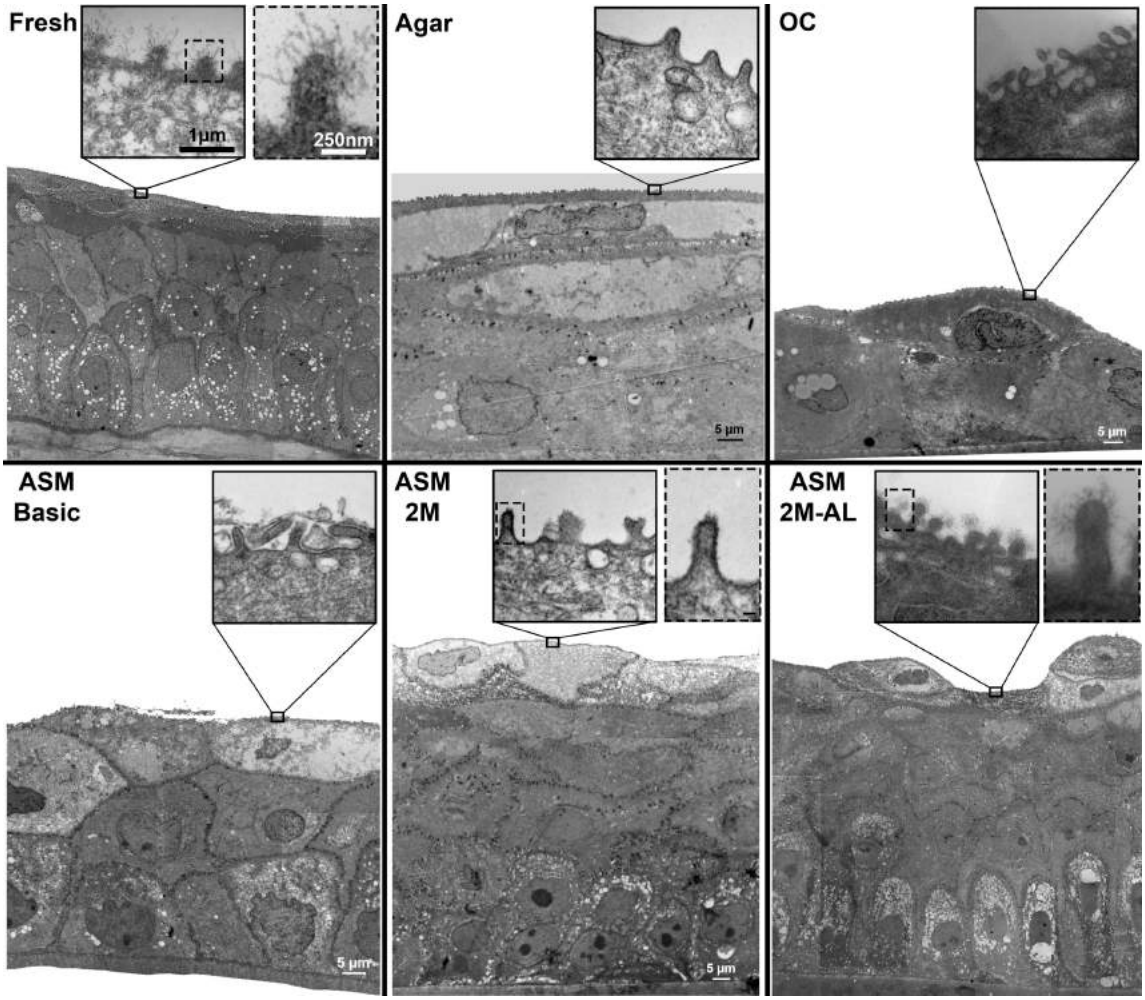



**Figure 5. Transmission Electron Microscopy (TEM) on central epithelium of human corneas after 14 days' storage in organ culture, on agar or in the active storage machine (ASM).**

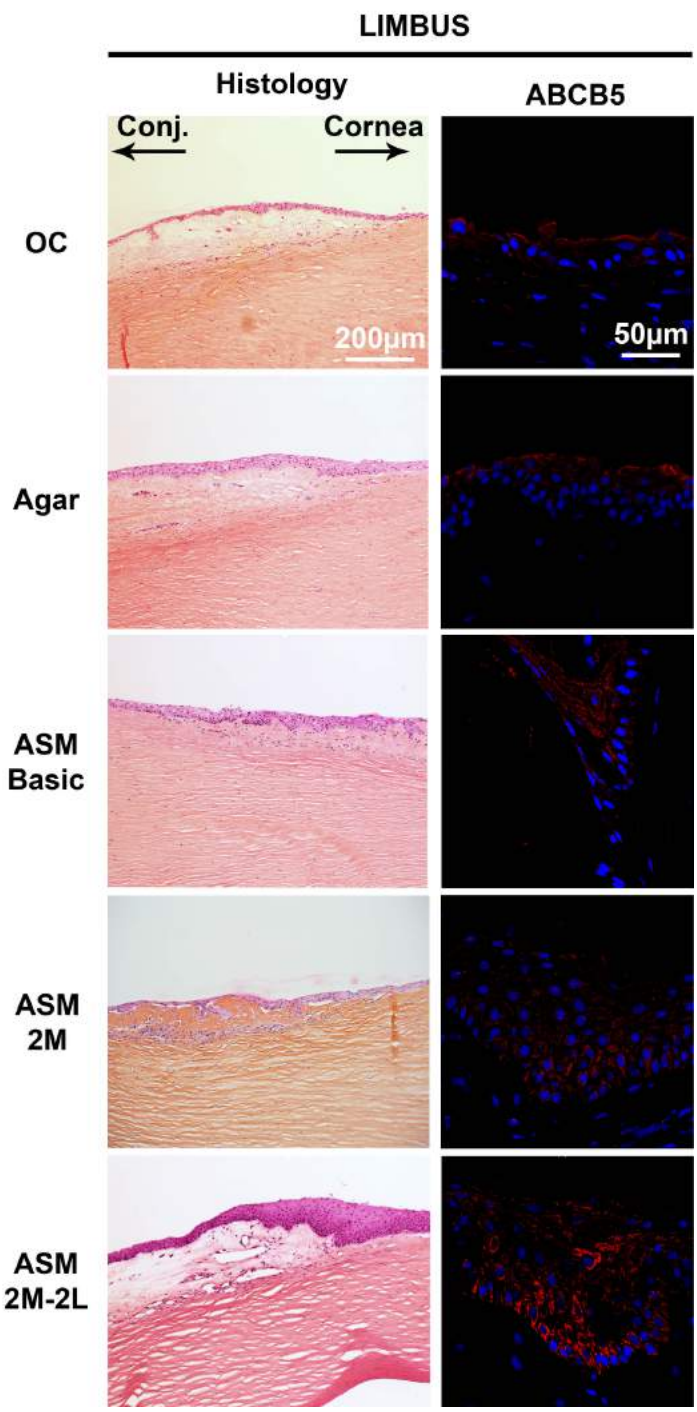



**Figure 6. Histologic cross sections (hematoxylin, eosin, and saffron) and immunostaining on sections of the limbal epithelium of human corneas after 14 days' storage in organ culture, on agar or in the active storage machine (ASM). ABCB5 (red). Nuclei were stained with TO-PRO-3 Iodide (blue). No staining was observed in negative controls (data not shown).**

**Table 2. Primary antibodies**

| Target protein | Animal source | Role (Expected Cell Compartment) | Reference and Manufacturer |
|---|---|---|---|
| ZO-1 | mouse | Tight junctions (apical plasmic membranes) | 40-2200. Zymed, Carlsbad, CA |
| ABCB5 | rabbit | Stemness (plasmic membranes) | NBP1-50547. Novus Biologicals, CO, USA |
| E-Cadherin | mouse | Epithelial cells (all plasmic membranes) | 33-4000. Thermo Fisher Scientific |
| Laminin-5 | rabbit | Basement membrane | AB14509. Abcam, Cambridge, UK |
| Cytokeratin 3 (K3) | mouse | Differentiated corneal epithelial cells (cytoplasmic) | Sc-80000. Santa Cruz Biotechnology, Dallas, TX, USA |
| Cytokeratin 12 (K12) | mouse | Differentiated corneal epithelial cells (cytoplasmic) | Sc-515882. Santa Cruz Biotechnology, Dallas, TX, USA |

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**Acknowledgement**

Supported by The National Agency for the Safety of Medicines and Health Products (L'Agence nationale de sécurité du médicament et des produits de santé or ANSM), appel à projet recherche 2012, BANCO project, the Fondation de l'Avenir pour le recherche médicale appliquée, AP-RM-16-008 project, la Fondation des Aveugles de Guerre, and the French National Research Agency (ANR-13-PRTS-0001).

**Disclosure:** PG and GT have patented the bioreactor (P). The other authors have no proprietary or commercial interest in any materials discussed in this article (None)

# Annexes 2: The OBSERV platform (Ophthalmic Bioreactor Specialized in Experimental Research & Valorization): Overview of possible applications