Eye (2008) 22, 1223–1232

www.nature.com/eye

CAMBRIDGE OPHTHALMOLOGY SYMPOSIUM

The rabbit as an animal model for post-natal vitreous matrix differentiation and degeneration

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Abstract

Purpose **This study evaluates whether rabbits are a suitable animal model to study post-natal vitreous differentiation and degeneration.**

Methods **Human and rabbit eyes of various ages were studied by complementary anatomical techniques, light microscopy, and transmission electron microscopy.**

Results **The global vitreous matrix organization is similar in human and rabbit eyes, lamellae are an important aspect thereof and show striking morphological changes with increasing age. In humans, liquefaction is more conspicuous than in rabbits but changes in matrix histology consistent with liquefaction can also be observed in the latter.**

Conclusions **Lamellar development is consistent with vitreous differentiation, while increasing liquefaction is consistent with matrix degeneration. At the anatomical and histological levels, human and rabbit vitreous matrices are sufficiently similar to make the rabbit a promising animal model for the study of the pathogenesis of vitreous matrix differentiation and degeneration in more detail.**

Eye (2008) **22,** 1223–1232; doi:10.1038/eye.2008.39; published online 7 March 2008

Keywords: human; rabbit; vitreous; liquefaction; differentiation; ageing

Introduction

The vitreous body is transparent, with an extremely high water content (99%), and thus only a low percentage of macromolecular building blocks (0.1%),[1] most importantly collagens,[2] hyaluronan,[3] proteoglycans,[4–6] and glycoproteins.[5,7,8] Despite their low concentrations, these macromolecules form a structured extracellular matrix with a cortical, intermediate, and central zone.[9,10] Concentrations of macromolecules vary between these regions.[1,11] Furthermore, specific anatomical structures have been defined within human and animal vitreous matrices.

The vitreous body is subject to an ongoing process of matrix remodelling, starting at embryogenesis and continuing into old age. During the embryonic period, three developmental stages are distinguished based on different morphological aspects of the matrix—the primordial, primary, and secondary vitreous.[12] Already, studies of rabbit vitreous have provided new insights into the relationships between these stages, in particular the latter two. Evidence has been found for a true remodelling of the matrix and the classic concept of appositional growth of the secondary and compression of the primary vitreous has been questioned.[13] This inherently questions the origin of Cloquet's channel, which is thought to represent the remaining and centrally displaced primary vitreous.[12] A point of distinction is the timing of the disappearance of the hyaloid system, which is prenatal in humans[14] and takes place in the first 2 weeks post-natal in rabbits.[15]

In post-natal life, dense structures start to develop within the vitreous matrix. In humans, these gradually become more conspicuous[10,16,17] and increasingly irregular on ageing.[10,17] At the same time, fluid-filled areas that grow in volume over time are observed within the matrix.[9,10] The earliest signs thereof are found in humans aged 4–5 years.[19] While the presence of dense structures and fluid-filled spaces is generally accepted, two opposing views are

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Received: 18 January 2008
Accepted in revised form: 18 January 2008
Published online: 7 March 2008

Proprietary interests: None

held as to their nature. The first interprets both as expressions of matrix destabilization, thus linking them pathogenically both in time and space.[17,18,20] The second distinguishes between a process of matrix differentiation resulting in the formation of dense structures, and a process of matrix degeneration resulting in the formation of fluid-filled spaces.[21] Histological evidence of collagen fibre fragmentation supports the latter theory.[21] Ultimately, a posterior vitreous detachment occurs. This is a phenomenon causally associated with vitreoretinal interface pathology.[22]

To study the formation of vitreous dense structures and liquefaction in more detail, as well as strategies that influence them, a suitable animal model is useful. This model must have dense vitreous structures with liquefaction forming on ageing. Furthermore, the animal should be easy to keep and young, adult, and older animals should be readily available. Rabbits are a practical animal model because of their size, eye volume, availability (at least at young and adult ages), and the limited period between birth and adulthood.[23] Obvious anatomical differences with the human eye include volumetric differences (a difference in the relative volumes of the lens and vitreous), the presence of vascular medullary rays in an otherwise avascular retina, and the presence of a visual streak in the absence of a true macula lutea.[24] Despite these shortcomings, the rabbit is the most commonly used animal model for the study of vitreous and retina pathologies and for the evaluation of the effects of medical and surgical interventions at these sites. This paper therefore evaluates whether the rabbit can serve as an animal model for the study of post-natal vitreous matrix remodelling in detail. The scope of this study is a comparison at the anatomical and histological levels. Ultimately, these data should be expanded with biochemical data on matrix components at various ages.

Materials and methods

Human donor eyes with no known ophthalmic disorders were obtained from the Cornea Bank (Amsterdam, the Netherlands) after the removal of the corneas for transplantation. Eyes were fixed by immersion within 48 h post-mortem and stored at 4°C. Rabbit eyes were obtained from the Centraal Dierenlab, Groningen and the Gemeenschappelijk Dierenlab, Utrecht (the Netherlands), where rabbits were killed for other, mainly non-ophthalmologic experiments. The ophthalmologic experiments did not involve the vitreous. Rabbits belonged to various breeds but were evaluated as a single group, since no differences in vitreous organization were observed between breeds. The age distribution between humans and rabbits differs. Rabbit vitreous could be studied at young, adult, and older ages, but old rabbits were hard to obtain. Human vitreous could be studied mainly from adulthood into old age, but only a limited number at childhood. Eyes were fixed by immersion within 1 h after death and stored at 4°C. In all eyes (human and rabbit), the 12 o'clock position was marked and left and right eyes were labelled as such.

Anatomy

Two complementary techniques were used[25]—(1) fixation in 0.25%. ruthenium red (RR; Fluka, Buchs, Switzerland) and 2% glutaraldehyde (GA; Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer (Fluka, pH 7.4) at 4°C followed by anatomical dissection to demonstrate variations in matrix density, and (2) fixation in 0.1% GA followed by careful blunt removal of the sclera, choroid, and retina. The retina was only removed where its attachments to the vitreous were loose; otherwise it was left in place to avoid damaging the vitreous. Dyes (Magic Colour, Royal Sovereign, London, UK) were injected to show areas with a very loose-meshed matrix structure or consisting of fluid-filled spaces.[25,26] Dyes are suspensions of particles with diameters of approximately 0.1–0.5 μm.[26] For both preparation methods, specimens were kept submerged in a buffer solution and were studied using a stereo microscope (Wild, type M-650). In total, 29 rabbit eyes from 21 rabbits (5–52 months) and 35 human eyes—of which 33 were from adults (21 donors, aged 17–87) and two from children (aged 5 and 7)—were studied.

Dyes were injected either into the vitreous base (at different clock hours) with the needle pointing in the direction of the optic disc, or into the preoptic area with the needle pointing towards the lens (Figure 1). Small

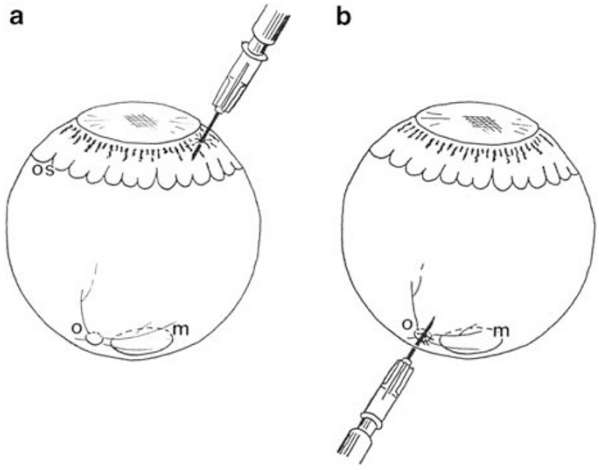

Figure 1 Dye injection technique. The dye is injected into the anterior vitreous (a) or into the Martegiani area (b). Abbreviations: o, optic nerve; os, ora serrata; m, macula.

quantities of dye were injected (varying between 0.1 and 0.4 ml) and the injection was stopped when the injection pressure need to be increased. After injection, the specimen was turned and the dye distributed itself over loose-meshed or fluid areas within the matrix in accordance with the laws of gravity. Injections into the preoptic area gave more consistent results and were therefore used in the majority of specimens.

Histology: light microscopy and transmission electron microscopy

Rabbit eyes ($n = 35$, 14 days to 52 months) and human eyes ($n = 2$, 16 and 23 years) were fixed in 0.1% GA in 0.1 M phosphate (Fluka) or sodium cacodylate buffer (pH 7.4). To increase the accessibility of the vitreous to washing and embedding procedures, small parts of the globe, consisting of the sclera, choroid, retina, and outer vitreous, were removed at 6 and 12 O'clock positions. Specimens were washed, dehydrated using ethanols (30–100%), and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) in low-temperature conditions (−20°C for infiltration and 4°C for polymerization). Sections 3–5 μm thick were stained with toluidine blue (Fluka) and evaluated using light microscopy (LM). Areas of interest were selected from the larger blocks for evaluation by transmission electron microscopy (TEM). Thin sections (90 nm thickness) were contrasted with uranyl acetate (Polyscience, Warrington, USA) in 25 cp methylcellulose (Sigma Chemicals, St Louis, MO, USA) and evaluated in a Philips (201) TEM operated at 60–80 kV.

All applicable institutional and governmental regulations concerning the ethical use of human and animal material were followed during this research.

Results

Anatomical dissection of RR/GA specimens

General organization

Adult rabbit and human eyes had a similar global matrix organization pattern—specifically, a more solid cortex could be discerned at the periphery, a fluid or very loose-meshed area at the centre, and an area of intermediate consistency in between. In both species, denser structures were observed to some extent in the cortex and more strikingly in the intermediate area (Figure 2). In both species, these dense structures had the appearance of extended sheets or lamellae. Therefore, we refer to them as lamellae. In sagittal sections, lamellae were seen to originate anteriorly from the vitreous base, which is the area between the bow region of the lens and the peripheral retina. They ran in the general direction of the posterior pole thus forming a funnel-like pattern (Figure 3). Differences between rabbit and human eyes were noted. In rabbits, lamellae could be seen to reach the posterior pole and to insert themselves at the optic disc. In human eyes, they could readily be discerned in the anterior and equatorial but not always in the posterior vitreous. Another difference between the two species was the organization pattern, which was very predictable in rabbits, but with some variation in the distribution patterns of lamellae in humans (Figure 3a and b). In humans, the funnel-like pattern predominated. Alternative patterns included S-shaped patterns and a backwards curving of the lamellae, either centred on the vitreous base area or centred on the equator of the eye.

In rabbits, differences in several aspects of the lamellae were observed between young and older animals. In young animals, lamellae were scarce, small, and ran a straight course. In older rabbits, lamellae were more

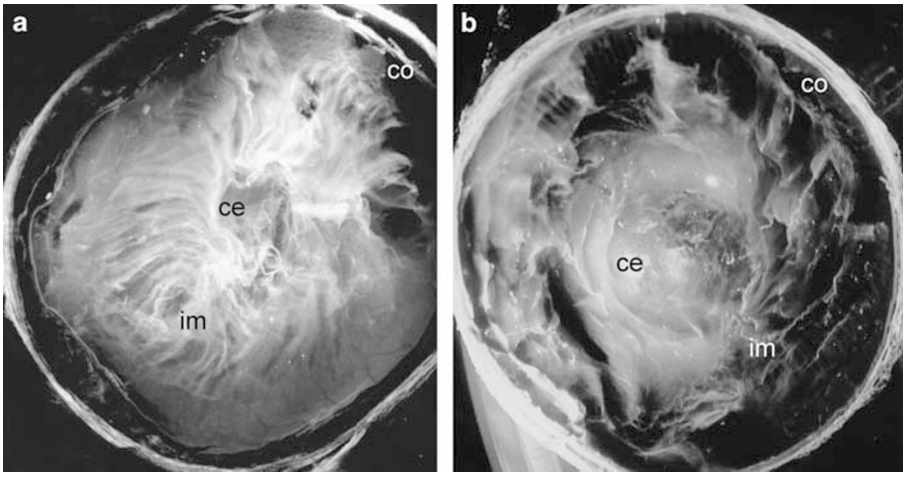

Figure 2 Frontal section through the equator of a ruthenium red/glutaraldehyde (RR/GA)-fixed human (a) and rabbit (b) vitreous showing a lamellar organization pattern most clearly in the intermediate vitreous (im) and cortex (co). Abbreviations: ce, centre; (figure (a) is reprinted with permission from *Experimental Eye Research*).[25]

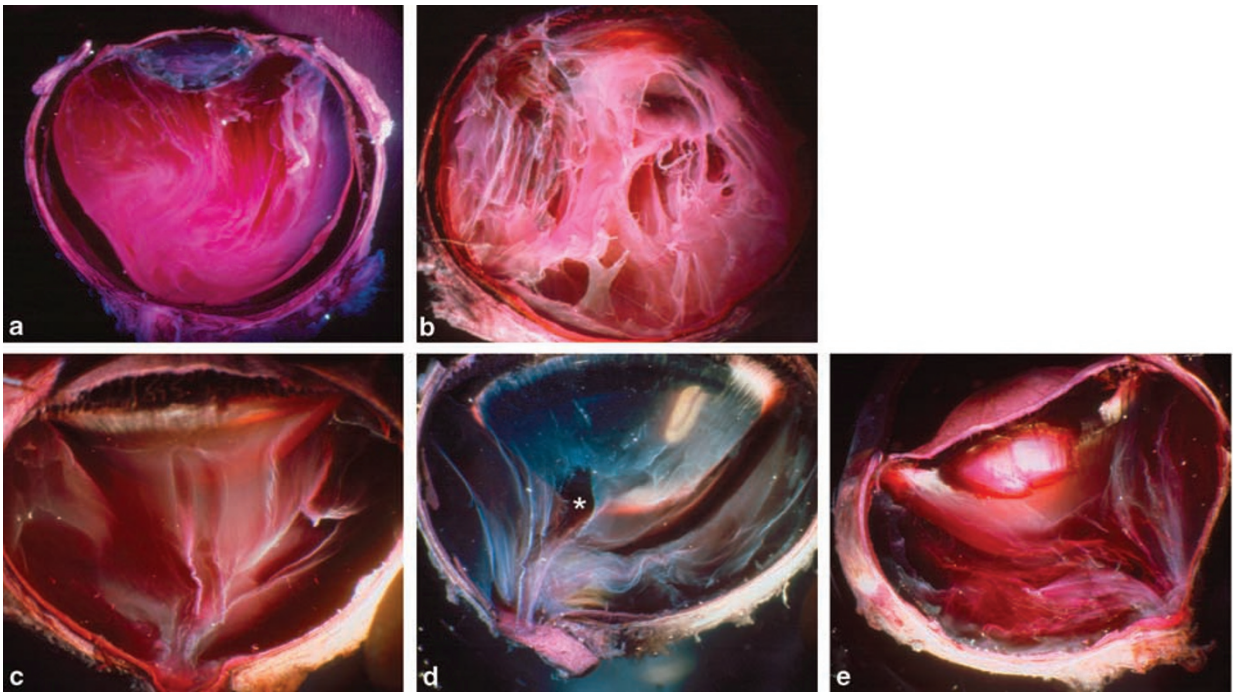

Figure 3 Sagittal section through a ruthenium red/glutaraldehyde (RR/GA)-fixed adult human (a, b) and rabbit (c–e) vitreous. (a, c–d) Funnel-like organization pattern of vitreous lamellae; (b) alternative organization pattern—lamellae curve backwards around the equator of the eye. (c–e) Upon ageing, an increase in the number and length of lamellae is observed—(c) 5–8 months, (d) 28 months, and (e) 43 months. (d) asterisk (*): Cloquet's channel. In all figures, the lens is at the 12 o'clock position (figure (a) is reprinted with permission from Kugler Publishers;[26] (d) is reprinted with permission from *Experimental Eye Research*).[25]

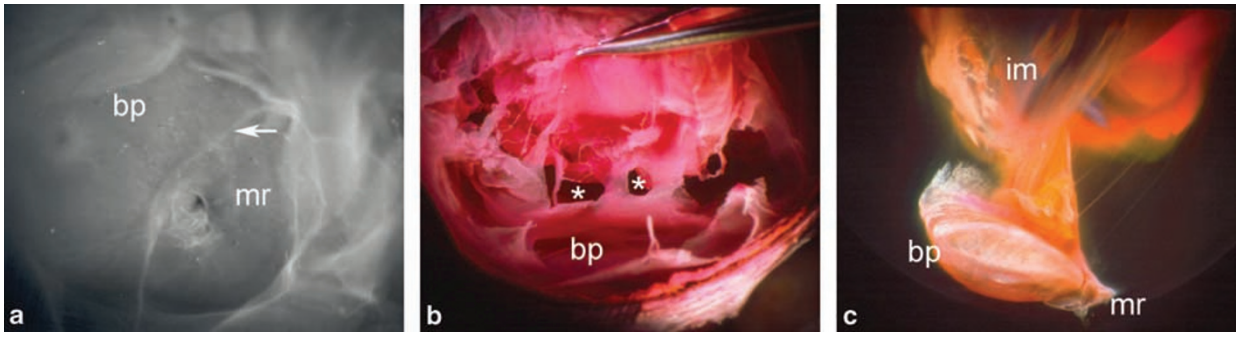

Figure 4 Posterior vitreous and flow area in adult human eyes. (a) Frontal section through a ruthenium red/glutaraldehyde (RR/GA)-fixed adult human eye. The section is made just below the roof of the bursa premacularis, thus revealing the base of the bursa premacularis (bp) and the adjacent Martegiani (mr) area, along with part of the wall separating the two (arrow). (b) Sagittal section through an RR/GA-fixed adult human eye. The bp is surrounded by a perforated wall (asterisks show a number of passageways), thus connecting this structure to the intermediate vitreous. (c) Dye injected into the Martegiani area in an adult (45 years) human eye, fills the mr, the bp, and the intermediate vitreous (im).

numerous, more conspicuous, broader, and undulating instead of straight (Figure 3c–e).

Specific structures

In rabbits, a central channel running from the optic disc towards the lens was clearly visible (Figure 3d). This structure is generally known as Cloquet's channel.[27] Wing-like lamellar structures, the 'alae canalis Cloqueti',[25] extend from this channel and insert themselves at the medullary rays. In humans, Cloquet's channel and its alae were not seen. In adult humans, a space surrounded by a thin membranous wall was observed in front of the optic nerve and another one in front of the macula (Figure 4a). They were incompletely separated by a thin perforated wall. These structures

were previously described by Martegiani[28] (Martegiani area) and Worst[29] (bursa premacularis), respectively. Multiple passageways existed connecting these spaces to the intermediate vitreous (Figure 4b). Such structures were never observed in rabbit eyes.

Dye injections

Humans

Dyes injected anteriorly into the vitreous base of adult human eyes almost invariably reached the posterior pole and filled the Martegiani area and the bursa premacularis. A dye could be injected at almost any clock hour position anteriorly and still reach the posterior pole. Therefore, no specific connections between any limited anterior regions and the posterior pole area were found. Dyes injected into the preoptic area filled the entire Martegiani area and bursa premacularis, and then, on rotation of the specimen, reached a large part of the vitreous base (Figure 4c). A dye injected at either site did not fill the retrolental area. Therefore, the area through which the dyes could flow originated anteriorly from a large part of the vitreous base, ran through the intermediate vitreous, and projected on the posterior vitreous at the Martegiani area and bursa premacularis.

Differences in the flow areas were observed when comparing young (5 and 7 years old), young adult (19–20 years old) and adult (31–47 years old) vitreous bodies (Figure 5a–d). Dye injected into the preoptic space of either of the children's eyes was seen to travel very slowly anteriorly with gravity. It took the dye 24–48 h to reach either the retrolental or the vitreous base areas. In only one of the eyes was a connection between the Martegiani area and the bursa premacularis found. In young adult eyes, dyes injected into the preoptic space always also filled the bursa premacularis and followed a limited number of passageways towards the anterior vitreous. The dye flowed slowly and reached the anterior extension of the passageways only after a few hours in a lens-down position. Some of the passageways reached the anterior vitreous, but others stopped in between the equator and the anterior vitreous. In adult eyes, the dye distributed itself much more rapidly (within minutes) and occupied large parts of the previously defined conical flow area.

Rabbits

Dyes injected into the preoptic space quickly filled Cloquet's channel (Figure 6a). Cloquet's channel was funnel shaped with its broader part projecting on the posterior lens capsule towards the 12 o'clock position. Dyes injected into Cloquet's channel remained confined

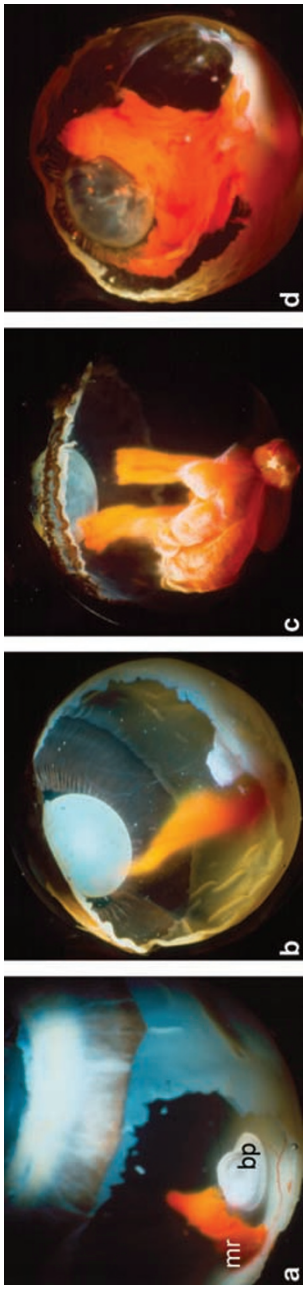

Figure 5 Age-related increase in the volume of the flow area in the human vitreous. (a, b) Child (7-year old): the bursa premacularis (bp) and Martegiani area (mr) are completely separated because the white and orange dyes do not mix. (b) The dye reaches the vitreous base area only after 48 h in a lens-down position. (c) Donor (18 years): after several hours in a lens-down position, the dye has filled a number of 'channels'. (d) Donor (47 years): the dye fills a large part of the intermediate vitreous within minutes.

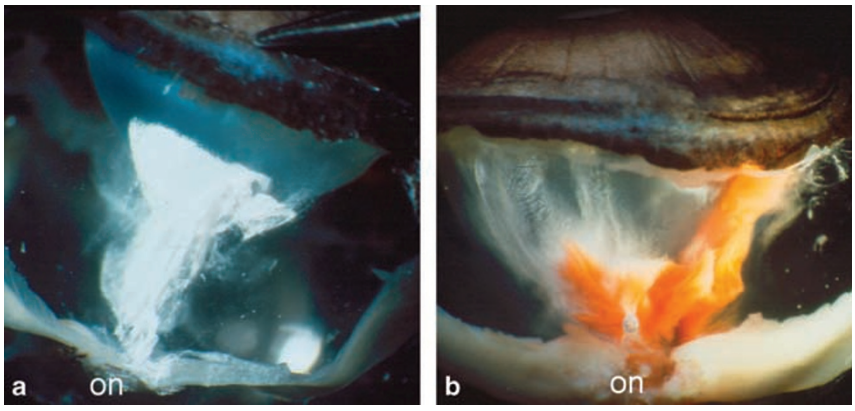

Figure 6 Rabbit vitreous body. (a) White dye injected into the preoptic vitreous fills Cloquet's channel and is strictly confined to this channel. (b) Orange/white dye injected into the vitreous base area fills a wider funnel surrounding Cloquet's channel. The funnel is subdivided into elongated segments by the presence of lamellae. Abbreviation: on, optic nerve (figure (a) is reprinted with permission from *Experimental Eye Research*).[25]

to this structure. Dyes injected into the vitreous base distributed themselves over a wider funnel surrounding Cloquet's channel; dyes were excluded from the denser lamellae that ran in the same area (Figure 6b).

Histology: light microscopy and transmission electron microscopy

We focused on the structure of lamellae compared to the surrounding matrix. In very young rabbits (0.5 months) indications of lamellae were observed as small numbers of mainly parallel-running fibres, forming a Y structure in sagittal sections (Figure 7a). These early lamellae were located at sites formerly occupied by hyaloid vessels and cells were observed alongside them. In young, adult, and older rabbits, the lamellae broadened and remained straight at first (Figure 7b), developing undulations later in life (Figure 7c). Lamellae stood out against the surrounding matrix by their denser packing of macromolecules and a more oriented—parallel—course of their fibrils. In young and adult rabbits, the matrix adjacent to the lamellae was regular in aspect. Only in the oldest rabbits was the matrix irregular and characterized by an aggregation of fibrils, the presence of fibril fragments, and prominent inter-fibrillar amorphous material (Figure 7c).

In adult humans, lamellae had an LM aspect similar to adult rabbits. Lamellae were dense structures embedded in a regular matrix. They had a mainly parallel fibre orientation when observed by LM and a fibre network aspect when evaluated by TEM (Figure 7d and e).

Discussion

This study demonstrated overall similarities in matrix structure between rabbit and human eyes. When comparing specific structures between both species, important differences were found.

A differentiated overall vitreous matrix structure as described previously[9,10] was confirmed in this study both in rabbits and in humans. Within the general pattern of a cortex, intermediate area, and centre, lamellar densities were present in all eyes but to a different extent. Lamellae mainly recognized in the intermediate vitreous and in the cortical area determined the anatomical pattern of matrix organization. This pattern was highly predictable in rabbit and somewhat less so in human eyes. The patterns observed in this study correspond to a large extent to the previously described organization patterns of vitreous densities in human eyes.[9,16] The variability in human vitreous organization observed in this study also helps to explain why different organization patterns for human vitreous have been described in the literature.[10,17,30,31] The strength of this study lies in the fact that the lamellae observed anatomically could be studied *in situ* by LM and TEM, thus revealing their histological aspects. They could also be observed in rabbits of various ages and thus reveal developmental aspects.

In rabbits, an interesting relationship between developing lamellae and former embryonic blood vessels was noted. Lamellae appeared at sites previously occupied by hyaloid vessels and were accompanied by cells, probably macrophages involved in the clearing of blood vessel remnants.[32,33] This suggests that lamellae are formed along tracts previously occupied by blood vessels, thus confirming earlier observations in human eyes.[34] In humans, lamellae gradually became more conspicuous and irregular on ageing.[10,16,17] Similar observations were made in rabbits, where lamellae first became broader over time and later started to undulate as well. This is suggestive of a process of lamellar growth

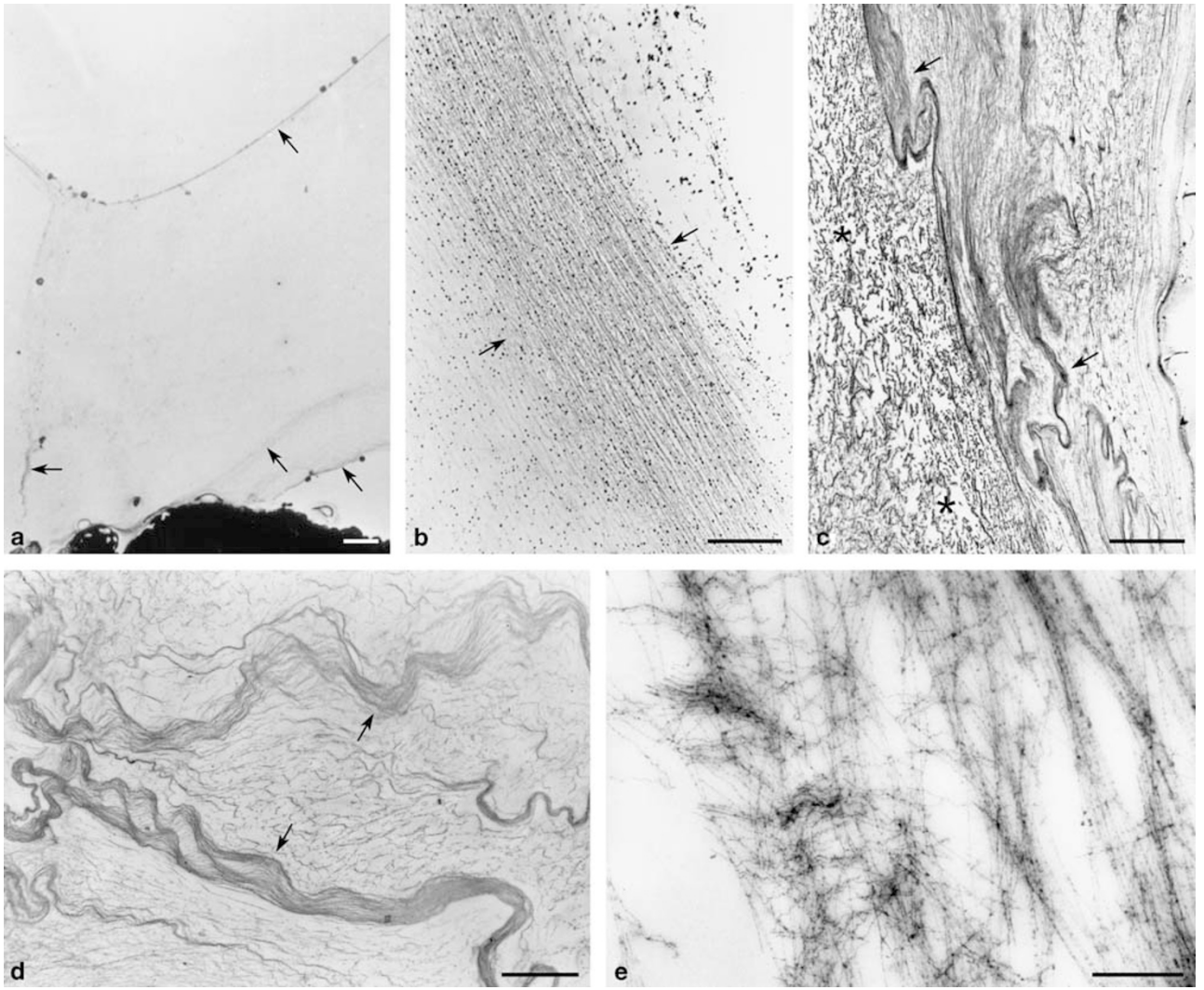

Figure 7 (a–c) Light microscopy (LM) overview of lamellae (arrows) in rabbits of various ages showing an increase in the width and length of lamellae upon ageing (bars = 100 μm): (a) 2 weeks, (b) 5–8 months, and (c) 47 months. The matrix adjacent to the lamellae has a regular aspect in (a) and (b), but is irregular in (c), in particular to the left of the lamellae (asterisks). (d–e) Lamellae (arrows) in adult human eyes. (d) LM: lamellae seem to consist of parallel-running fibres (bar = 100 μm) and are embedded in a normal-seeming matrix; (e) Transmission electron microscopy (TEM): lamellae appear to consist of fibre networks rather than parallel-running fibres alone (bar = 1 μm) (figures (b) and (c) are reprinted with permission from *Experimental Eye Research*).[25]

by the continuous accretion of at least collagenous and probably also other macromolecular matrix components. Lamellae seem to consist of parallel-running fibres when viewed by LM, but appear to consist of fibre networks after TEM.[25] Therefore, lamellae and the surrounding matrix have an essentially similar network structure, the main differences being mesh diameter and predominant fibre orientation.

The formation of lamellae along predefined tracts and their growth on ageing would be consistent with a process of matrix differentiation rather than matrix degeneration. The fact that lamellae are surrounded by a regular matrix in young and adult rabbits, and only become embedded in a degenerating matrix at a much later age, confirms this. The irregular matrix observed in ageing rabbits resembles the liquefying matrix found in humans to a large extent.[21]

An important difference relates to the anatomy of the central vitreous. In rabbits, Cloquet's channel was always observed.[27] It was always located exactly between the optic disc and the posterior lens capsule. It was clearly separated from the surrounding matrix, and no significant increase in its volume was observed in adult rabbits at different ages. In observation by LM, it is surrounded by a regular matrix.[25] These aspects are most consistent with a tissue specialization. Our data indicate that Cloquet's channel develops between 2 weeks and 5 months of age in rabbits. This limited time frame permits the study of this process in detail in rabbits. In humans, the central retrolental vitreous had characteristics of a loose-meshed matrix, but it was not continuous with the Martegiani area. Therefore, we cannot confirm that Cloquet's channel is present in the human eye. Instead, the intermediate human vitreous contained a flow area, gradually increasing in volume on ageing and extending itself towards the bursa premacularis and the Martegiani area posteriorly, and the retrociliary area anteriorly. In all human specimens, a prepapillar and premacular space consistent with the previously described Martegiani area[28] (Martegiani) and bursa premacularis[29] could be identified. They were already observed at very young ages and have predictable locations. Unfortunately, we were unable to reveal the histological aspects of their walls. Therefore, it remains unclear whether they represent areas of matrix differentiation or areas of very early matrix degradation. The real existence of these structures was recently confirmed *in vivo*.[35] The flow area itself could be consistent with an area of matrix liquefaction or degradation. Arguments in favour of this include its gradual increase in volume on ageing and its variable extensions and borders consisting of irregular walls containing collagen fragments.[21]

Because of the similarities between human and rabbit eyes in post-natal matrix differentiation and degeneration, it appears that rabbits could be a suitable animal model to study both processes in more detail. With regard to vitreous differentiation, issues of interest would include the identification of the cells producing vitreous collagen postnatally, and processes determining the secretion and incorporation of new collagen molecules into the existing matrix. A logical candidate region would be the posterior ciliary body, as was already shown in various animal studies.[36–38] An anteriorly located collagen production site is also suggested by the finding of very high collagen concentrations in the anterior adult rabbit vitreous.[39] With regard to matrix degeneration, it would be necessary to identify matrix breakdown products by immunohistochemical and biochemical methods in both species and then to search for the enzymes involved in this process. Enzymes possibly involved include matrix metalloproteinases, a number of which have already been identified in the human vitreous.[40–43] Collagen breakdown in cartilage, a tissue biochemically similar to vitreous, is facilitated by a loss of proteoglycans,[44,45] a factor that may also apply to the vitreous, since an age-related loss of type IX collagen, which is also regarded as a proteoglycan, has already been described.[46]

Conclusions

Vitreous differentiation and degeneration are two different ongoing processes starting at a very young age and determining the structural changes observed on ageing. Immunohistochemical and biochemical studies are needed to better understand the physiology and pathophysiology of these processes. Knowledge in these fields would potentially provide us with tools to influence these processes and modify the process of age-related liquefaction.

Rabbits are a promising animal model for such study.

Acknowledgements

The study was financially supported by the Rotterdamse Vereniging Blindenbelangen, Haagsch Oogheelkundig Fonds, and Algemene Nederlandse Vereniging ter Voorkoming van Blindheid. The Cornea Bank (Amsterdam, the Netherlands) provided the human eyes, the Centraal Dierenlab (Groningen, the Netherlands) and the Gemeenschappelijk Dierenlab (Utrecht, the Netherlands) provided the rabbit eyes. The line drawings were made by G van der Veen, and M van Deemter digitized the illustrations. The anatomical studies were performed at the Jan Worst Research Group (Haren, the Netherlands) and the histological studies at the Laboratory for Cell Biology and Electron Microscopy (Groningen).

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