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Ultrastructural morphology and morphometry of the normal corneal endothelium of adult crossbred pig

Morfologia ultraestrutural e morfometria do endotélio corneal normal de suínos adultos mestiços

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ABSTRACT

Corneal endothelium constitutes a monolayer of polygonal cells. The integrity and health of this layer are essential for the maintenance of normal corneal transparency. This study reported by the first time in a detailed way the ultrastructural morphology and morphometry of the corneal endothelium in normal adult crossbred pigs by using scanning electron microscopy (SEM). A regular pattern of polygonal cells, with predominantly hexagonal cells and clear cell borders, was observed. An oval nucleus that bulges in the centre of the cell, cilia (2-4) in a few peripheral cells, openings of the pinocytotic vesicles, microvilli, borders bars and interdigitated cell borders were observed. The mean endothelial cell area was significantly higher (P<0.05) in the centre than in periphery, with a lower variation coefficient in the former. The mean cell density in periphery was significantly higher (P<0.05) than in centre and 43.9% higher than data formerly reported by other authors using specular microscopy, showing the significant shrinkage caused by sample processing for SEM. The mean value of cell sides (pleomorfism) was 5.9, ie. predominant hexagonal shape. The percentage of hexagonal cells was significantly higher in central region (P<0.01), determining a more homogeneous structure. The parameters obtained in this study will be a basement for future investigations about the effect on pig corneal endothelium of drugs, intracamerular surgeries and storage solutions for transplants.

Key words: *cornea endothelium, pig, scanning electron microscopy.*

RESUMO

O endotélio corneal é uma monocamada de células poligonais. A integridade e saúde dessa camada são essenciais para a manutenção da transparência corneal normal. Este estudo reportou pela primeira vez, de forma detalhada, a morfologia ultra-estrutural e a morfometria do endotélio corneal de suínos adultos mestiços à microscopia eletrônica de varredura (MEV). A superfície endothelial corneal apresentou um padrão regular de células poligonais, com predomínio da forma hexagonal e de bordas celulares nítidas. O núcleo foi observado como protuberância arredondada no centro da célula. Também foram observados os cílios (2-4) em apenas algumas células da região periférica da córnea, as aberturas das vesículas pinocitóticas na proximidade dos cílios, as microvilosidades, as varas da borda e as bordas celulares em formato de zigzag. A área celular média foi significativamente maior (P<0,05) no centro da córnea do que na periferia, com um coeficiente de variação menor no centro da córnea. A densidade celular média foi significativamente maior na periferia (P<0,05) e 43,9% maior que os dados reportados por outros autores na microscopia especular, o que demonstra o efeito da retração celular durante o processamento das amostras. O valor médio do número de lados das células (pleomorfismo) foi de 5,9, o que evidencia um predomínio do formato hexagonal. A percentagem de células hexagonais foi significativamente maior no centro (P<0,001). Os parâmetros obtidos nesta pesquisa servirão de base para estudos futuros sobre o efeito de medicamentos, cirurgias intracamerulares ou soluções para armazenamento de córneas para transplantes no endotélio corneal do suíno.

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Palavras-chave: *endotélio corneal, suíno, microscopia eletrônica de varredura.*

INTRODUCTION

The corneal endothelium constitutes a monolayer of polygonal cells. The integrity and health of this layer are essential for the maintenance of normal corneal transparency. Endothelial physiological alterations lead to oedema and loss of transparency (HOPPENREIJS et al., 1996). A lot of factors can affect its physiology, like drugs and surgical procedures (WARING et al., 1982).

Endothelial cell populations decrease with age and lesions, resulting in a significantly larger size and rounded shape of individual cells and leading, finally, to fluid filtration into the stroma and increase in corneal thickness (CARLSON et al., 1988; GULLAPALLI et al., 1982; GWIN et al., 1982; WILSON & ROPER-HALL, 1982; LAULE et al., 1978; LAING et al., 1976). Therefore, there is a correlation between endothelial morphology and its functional capacity (LANDSHMAN et al., 1988; YEE et al., 1987; SCHULTZ et al., 1986).

There are few morphological and morphometric studies on pig corneal endothelium (VICENTI, 2004; COLLIN & COLLIN, 1998; BAHN et al., 1986; NEUBAUER et al., 1984), despite this animal plays a model for experimental studies in ophthalmology. VICENTI (2004) and BAHN et al. (1986) studies quantified the normal corneal endothelium by specular microscopy (SM). NEUBAUER et al. (1984) reported an investigation on morphological effects of short-term storage using SM. Only COLLIN & COLLIN (1998) used scanning electron microscopy (SEM) for an ultrastructural study.

The ultrastructure and morphometry of the corneal endothelium in normal adult crossbred pigs were investigated using SEM to obtain theorical and practical basis for future studies in experimental and comparative ophthalmology (ie. effect of drugs, intracamerular surgeries and storage solutions for transplants).

MATERIALS AND METHODS

Five female and 5 male, 115 days old, clinically health crossbred Landrace-Large White pigs, weighting about 100kg, were used. The eyes were enucleated and put in 2.5% glutaraldehyde with the cornea up. Corneal buttons were obtained by a 360º section 2 to 3mm from the limbic region to the sclera and reduced to 4 portions.

Sample preparation for SEM was done following the report of VIRTANEN et al. (1984). Samples were transferred to a 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.4 and stored for 24h at 4ºC for fixation. Then they were washed in the buffer, and post fixed for 2h in 2% osmium tetraoxide in 0.1 M cacodylate buffer at 4ºC. Subsequently, the corneas were washed again in cacodylate buffer and dehydrated through an increasing series of ethanol solutions. Thereafter, the specimens were submitted to critical point drying using liquid carbon dioxide. Corneas were placed on 10mm aluminium stubs with double-sided adhesive tape and sputter coated with gold-palladium.

The posterior endothelial surfaces were photographed by scanning electron microscopy (JEOL-JSM 5410, Tokyo, Japan) operating at 15kV. Ten photomicrographs of central corneal endothelium and 10 of peripheral corneal endothelium at x750 were obtained from each cornea and scanned. The cell area of 100 endothelial cells from each cornea was estimated using the computer-assisted image analyzer *Image Pro-Plus*® (Media Cybernetics, Silver Spring, Md). The mean cell area, mean endothelial cell density, percentage of variation in polygonality and the coefficient of variation of mean cell area of corneal endothelial cell were estimated. Finally, ultrastructural characteristics were described using photomicrographs obtained at x3,500 and x7,500. Statistical data analysis was conducted using the *F* test. Values of $P<0.05$ were considered significant (SAS, 1996).

RESULTS

A regular pattern of polygonal cells, with predominant hexagonal shape, and clear cell borders was observed (Figure 1). An oval nucleus that bulges in the centre of the cell, cilia (between one to four per cell), openings of pinocytotic vesicles, microvilli, border bars and border cell with zig-zag appearance due to the interdigitations, were observed. Cilia were present only in few peripheral cells and close to them there were openings of pinocytotic vesicles like small pits. Microvilli were present in the surface of all of the endothelial cells like numerous white points. Border bars were present in the boundaries of the cells, localized in right angles (Figure 2).

Mean cell area value for central cornea was $130.7\pm11.6\mu m^2$ (values range: 104.1-$154.3\mu m^2$, coefficient of variation [CV] 8.9%), and for peripheral cornea was $127.6\pm12.3\mu m^2$ (values range: 103.1 e $151.7\mu m^2$, CV 9.6%). There was statistically significant difference between centre and periphery values ($P<0.05$). A coefficient of cell shrinkage of 44% was

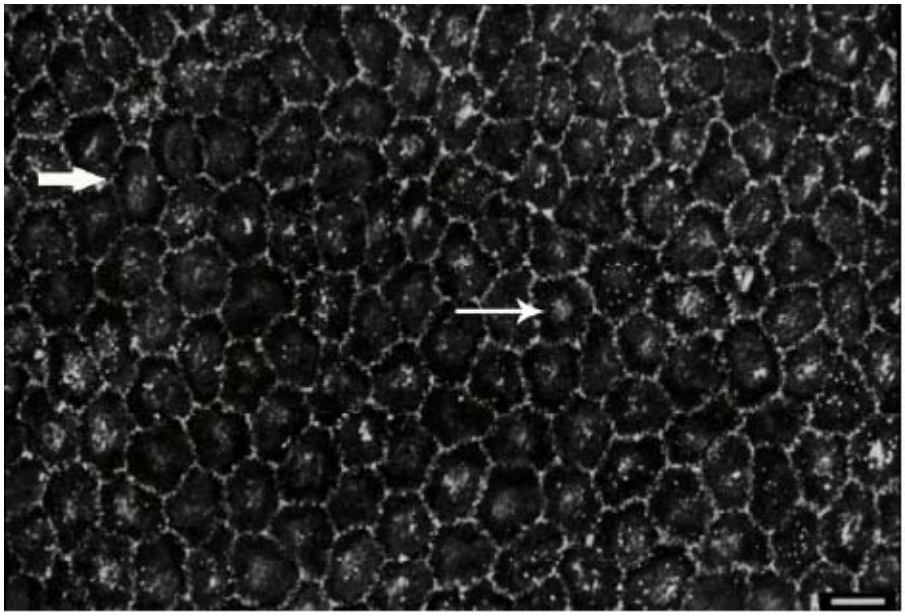

Figure 1 - Scanning electron micrograph of healthy crossbred swine cornea. A regular pattern of polygonal cells is observed, with predominant hexagonal shape and clear border cells (wide arrow). Nucleus is seen like a rounded bulged in the centre of the cell (thin arrow). Scale Bar: 10µm.

calculated dividing the mean cell area by SEM (present results) with the mean cell area by SM (results reported by VICENTI [2004] for swine) (129.2µm^2/293.4µm^2).

Mean cell density was 7,625.2±998.2 cells mm^{-2} (values range: 6,482.7 e 9,610.9cells mm^{-2}, CV 13.1%) in central cornea, and 7,909.7±776.3cells mm^{-2} (values range: 6,714.3 e 9,701.8cells mm^{-2}, CV 9.8%) in peripheral cornea. There was a statistically significant difference between central and peripheral cornea ($P<0.05$).

Regarding polygonality of the endothelium (pleomorfism), the majority of cells were six-sided (61.7±8.5% for centre and 53.2±7.0% for periphery), with the presence of five- (21.3±5.8% e 28.9±5.1%, for centre and periphery, respectively) and seven-sided (11.0±6.4% for centre and 18.1±4.8% for periphery) for the remaining cornea. There was statistically significant difference between central and peripheral cornea for six-sided and seven-sided cells values ($P<0.01$). Average number of sides per cell was 5.9 (Figure 3).

It was not detected any statistically significant difference among male and female, or among right and left eyes for any of the estimated parameters.

DISCUSSION

A regular pattern of polygonal cells was observed, with predominant hexagonal cells, similarly to data previously reported for other mammals (rat, rabbit, dog and human) (YEE et al., 1987). Pinocytotic vesicles openings were observed, with similar characteristics to previous reports, ie. small pits in the posterior and lateral cell surface, as described in humans and monkeys by SVEDBERGH & BILL (1972). These vesicles show the high metabolic activity endothelial cells have, which is required for the maintenance of corneal transparency.

The presence of cilia in corneal cells has been reported in various vertebrates (COLLIN & COLLIN, 1998 [birds]; BEUERMAN e PEDROZA., 1996 [humans]; GALLAGER, 1980 [rabbit]; SVEDBERGH & BILL, 1972 [humans and monkeys]), but not in pigs. In the present study one to four cilia were found only in peripheral cornea, in agreement with previous reports in humans and monkeys (SVEDBERGH & BILL, 1972). However, GALLAGER (1980) described cilia in every endothelial cell of the rabbit cornea and suggested that cilia are easily broken off during mounting procedure and that they retract under physiological stress, which could be a reason for the absence of cilia in the central cornea in the present study.

Microvilli, border bars and interdigitated cell borders were observed, which is in agreement with the investigations of COLLIN & COLLIN (1998) in four vertebrates classes (Teleostei, Reptilia, Aves and

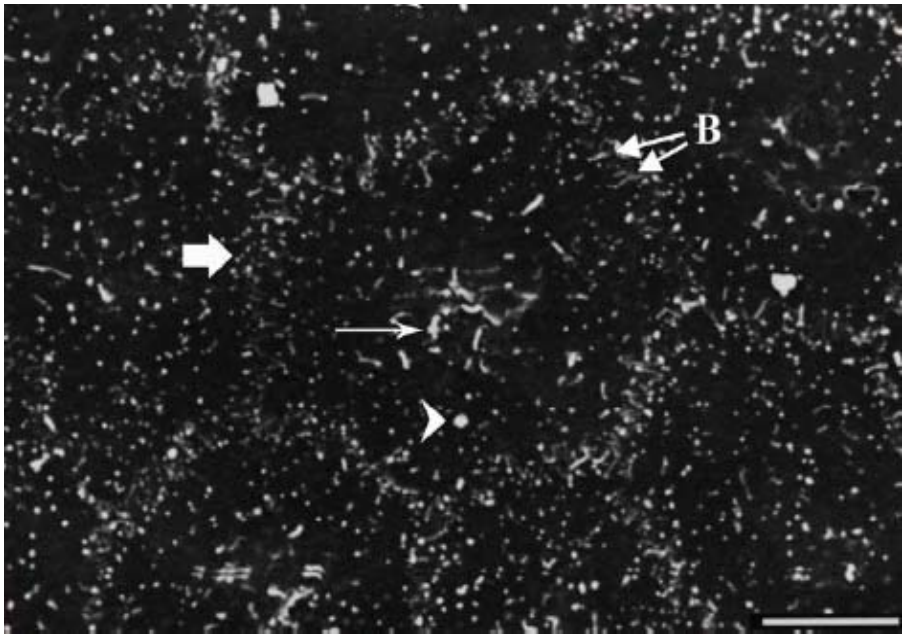

Figure 2 - Scanning electron micrograph of peripheral healthy crossbred swine cornea. Cilia (thin long arrow), microvilli in the cell surface (head arrow), cell borders in zig-zag appearance (wide arrow), openning of the pynocitotic vesicles close to the cilia (short thin arrow) and the border bars (V). Scale Bar: 1μm.

Mammalia). BEFANIS et al. (1981) observed the same in dogs and SVEDBERGH & BILL (1972) in man and monkeys. Hassall-Henle warts were not found. SVEDBERGH & BILL (1972) reported that such warts were present in animals with advancing age and indicated risk of corneal decompensation; therefore, the lack of them here observed is probably because the animals were young adults with normal eyes.

Regarding mean cell area, we found statistically significant higher values for central cornea (130.7±11.6μm^2 [CV 8,9%] against 127.6±12.3μm^2 for periphery [CV 9.6%]), while VICENTI (2004) reported significant higher values for peripheral cornea (289.9μm^2 for centre and 296.8μm^2 for periphery [P<0.01]). Similar data was reported by BINDER et al. (1979), in human corneal endothelium by SM and SEM, and by LAULE et al. (1978), in human corneal endothelium by SM. Coefficient of variation of mean cell area was lower in central cornea, showing that this region is more homogeneous, in agreement with the study of BLACKWELL et al. (1977) in humans (CV 31% for central and 43% for peripheral cornea).

A statistical significant difference between central and peripheral corneal cell densities was found in this study, with higher values for periphery (7,625.2±998.2cells mm^{-2} for centre [CV 8.9%], and 7,909.7±776.3cells mm^{-2} for periphery [CV 9.6%]). These results are in agreement with those reported by IRVINE for humans (1956) (2,881±175cells μm^{-2} for centre and 2,356±153cells μm^{-2} for periphery) and in contrast with VICENTI (2004) for pigs (3,460cells μm^{-2} for centre and 3,380cells μm^{-2} for periphery).

Our results showed a smaller mean cell area and greater density values in comparison to the values reported by VICENTI (2004), which can be explained by the shrinkage caused by the processing of SEM. The calculated cell shrinkage coefficient (44%) is similar to the one previously reported by BINDER et al. (1979) (31%, ranging from 5 to 50%).

Polygonality values (pleomorfism) found in this study (61.7±8.5% in the centre and 53.2±7.0% in periphery for six-sided, 21.7±5.8% in the centre and 28.9±5.1% in periphery for five-sided, and 11.0±6.4% in the centre and 18.1±4.8% in periphery for seven-sided cells) were similar to the reported by LANDSHMAN et al. (1988) in cats (66.5% for six-shaped cells), by VICENTI (2004) in pigs (52, 27 and 21% for six-, five- and seven-sided cells) and by GULLAPALI et al. (1982) in humans (48, 25 and 15% for six-, five- and seven-sided cells). None of these authors reported the specific values for the centre and the periphery of the cornea. In contrast with the results reported by

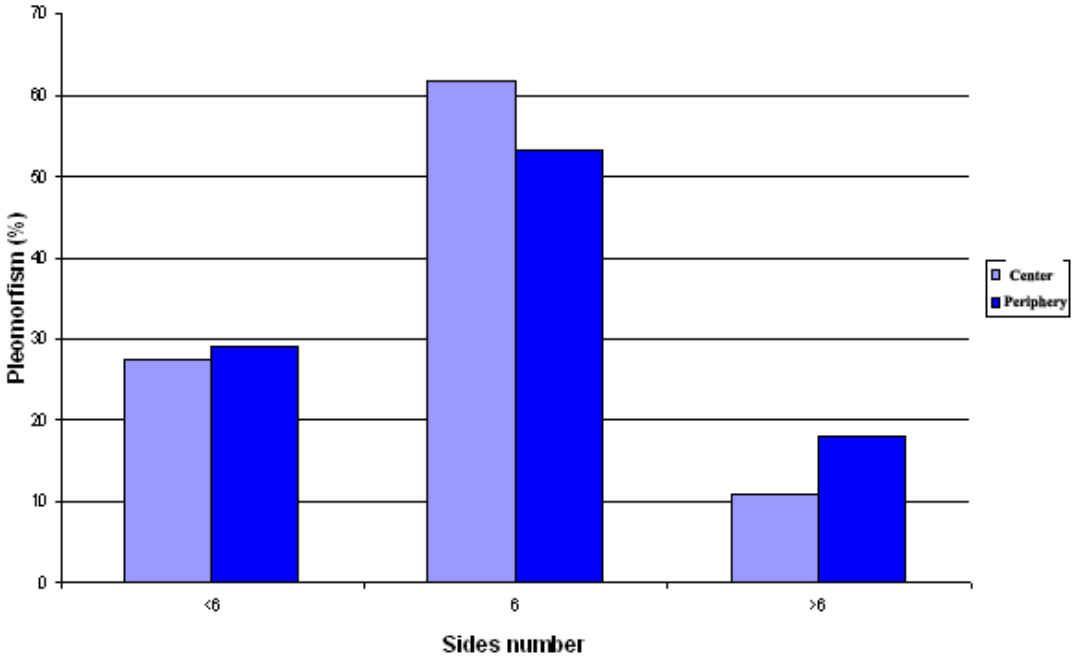

Figure 3 - Histogram of pleomorfism of endothelial cells in central and peripheral cornea in healthy crossbred swine. Media values ± standard deviation. n = 10. * Statistically significant difference (P<0.01).

VICENTI (2004), we found a statistically significant difference between central and peripheral cornea for this parameter. A decreased polygonality in damaged corneas has been reported (LANDSHMAN et al., 1988), which shows that this parameter could be an indicator of the corneal health status. Our results showed endothelial cells with minimal variation in size, probably due to the fact that all animals were the same age, and only healthy corneas were used.

There were no significant differences among eyes, and between male and female for any parameter, which is in agreement with previous reports in pigs (VICENTI, 2004), horses (ANDREW et al., 2001), cats (HUANG et al., 1989), dogs (GWIN et al., 1982), and humans (BLACKWELL et al., 1977).

Swine species is one of the best models for recreating some human diseases and also for investigations about cornea transplantation. The authors of this paper hope that this research would help in future investigations for the advancement of these topics.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All procedures were performed in compliance with the Association for Research in Vision and Ophthalmology statement on the use of animals in ophthalmic and vision research, and approved by the bioethics and biossecurity committee of the São Paulo State University (UNESP) (Nº. 0128-2004).

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