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Corneal endothelial cell density and morphology in rabbits' eyes using contact specular microscopy

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ABSTRACT: *The goal of this study was to describe the normal values of corneal endothelial cell density and hexagonality in healthy rabbits' eyes using contact specular microscopy. Both eyes of 18 rabbits, males and females, of different ages were evaluated. The eyes were divided into three groups of 12 each according to the age range of the animals: G1 (6 months old), G2 (12 months old), and G3 (48 months old). Parameters studied included mean endothelial cell density and hexagonality. For Group I, the mean cell density was 2307 cells per mm². For GII, the mean cell density was 1895 cells per mm². For G III, the mean cell density was 1818 cells per mm². Cell density decreased significantly with aging (P<0.001) among the three groups. Pleomorphism in the rabbits of GI was 74.33±10.08. In the rabbits of GII it was 71.83±11.38. In the rabbits of GIII it was 64.02±28.80. Significant differences (P<0.001) were evident between GI and GIII and GII and GIII but not between Groups I and II. The results showed a decrease in number of cells and hexagonality with aging. Corneal endothelium of rabbits suffers changes due to advancing age both in terms of cell density and morphology.*
Key words: *lagomorphs, corneal endothelial hexagonality, specular microscope.*

Densidade celular e morfologia das células do endotélio da córnea de coelhos obtidas com microscopia especular

RESUMO: *Objetivou-se avaliar a densidade celular e a hexagonalidade do endotélio da córnea de coelhos saudáveis usando um microscópio especular de contato. Os dois olhos de 18 coelhos, machos ou fêmeas, de diferentes idades foram avaliados. Os olhos foram separados em três grupos de 12 olhos cada em função da idade: G1 (6 meses de idade), G2 (12 meses de idade) e G3 (48 meses de idade). Os parâmetros estudados incluíram a densidade celular e a hexagonalidade. Para o Grupo I, a densidade celular média foi de 2307 células por mm². Para GII, a densidade celular média foi de 1895 células por mm². Para G III, a densidade celular média foi de 1818 células por mm². A densidade celular diminuiu significativamente com o envelhecimento (P<0,001) entre os três grupos. O pleomorfismo nos coelhos de GI foi de 74,33±10,08. Nos coelhos do GII foi 71,83±11,38. Nos coelhos de GIII foi de 64,02±28,80. Diferenças significativas (P<0,001) foram evidentes entre GI e GIII e GII e GIII, mas não entre os Grupos I e II. Os resultados revelaram diminuição da densidade celular e da hexagonalidade com o avanço da idade. O endotélio da córnea de coelhos sofre alterações devido ao avanço da idade tanto na densidade celular e quanto na morfologia.*
Palavras-chave: *lagomorfos, hexagonalidade, endotélio da córnea, microscópio especular.*

INTRODUCTION

The corneal endothelium is a single layer interlocking multi-sided cells making up the most posterior layer of cornea. Endothelial layer is of fundamental importance in maintaining the transparency of the cornea and changes in endothelial cells can occur due to aging, eye diseases, drugs or intraocular surgery. Specular microscopy is among the most reliable techniques for analysing the corneal endothelium (ABIB & BARRETO, 2001; NAGATSUYU et al., 2014). Normal endothelial parameters have already been established in some species using a specular microscope, including: humans, sheep, dogs, monkeys, horses, cats, and others (ABIB & BARRETO, 2001; ANDREW, S. et al. 2001; PIGATTO et al., 2006; PIGATTO et al., 2008; FRANZEN et al., 2010; BERCHT et al., 2015; COYO et al., 2016; TERZARIOL et al., 2016). Furthermore, specular microscopy is beginning to be used as part of the ophthalmic examination before and after cataract removal procedures in dogs (NAGATSUYU et al., 2014).

Due to the existence of interspecies variations in endothelial parameters, knowledge of normal data on the endothelium in each species is necessary. Previous studies have shown that rabbit corneal endothelium repairs by cell division and

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migration (MORITA, 1995). Rabbits have been widely employed in the evaluation of the effects of surgical procedures and drugs on the corneal endothelium (ATILLA et al., 2003; MENCUCCI et al., 2005; ARI et al., 2015). However, there are few published references regarding endothelial density in rabbits. SAILSTAD & PEIFFER, (1981) evaluated corneas from healthy rabbits using specular microscopy and reported that average number of cells for rabbits was 2998 cells per mm^2. MORITA (1995) reported that endothelial density decreases in rabbits over 12 months old. Moreover, there are no data regarding the effect of aging on corneal endothelial hexagonality in rabbits. The knowledge of the normal endothelial parameters is important not only for clinical evaluation but also for assisting in the planning of future studies related to corneal transplantation in rabbits. This study was carried out with the objective of evaluating the cell density and cell morphology of the corneal endothelium of rabbits of different ages using contact specular microscopy. In addition, it aimed to evaluate if there is a difference in the endothelial parameters in relation to the age of the animals.

MATERIALS AND METHODS

Thirty-six healthy eyes from 18 New Zealand white rabbits, males and females, obtained from the São Nunca rabbitry (Araricá, RS, Brazil) were used in this study. Rabbits died of death from natural causes for reasons unrelated to this study and died of disease that did not directly affect the eye. All stages of the study were performed according with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in studies related to ophthalmology. Enucleation was performed within 4 hours of death. Ophthalmic examination was performed immediately after enucleation. The examination consisted of biomicroscopy with slit lamp (Portable Slit Lamp SL 15, Kowa, Japan) and fluorescein test (Fluorescein strips, Ophthalmos, SP, Brazil). Each group consisted of 12 eyes. Group I comprised rabbits aged 6 months, Group II comprised rabbits aged 12 months, and Group G III comprised rabbits aged 4 years. Eyes were studied immediately after enucleation and kept in a wet chamber. The eyes were taken to the specular microscope and examined using a contact specular microscope (Celmax, Medical Service, Brazil) with software available for endothelium analysis (Specular Corneal Microscopy, Celmax). All eyes were submitted to specular microscopy and those who had some alteration were not included in the study. From each sample, three clear images were captured. In each image at least 60 endothelial cells were analysed. All evaluations were performed by the same investigator. Parameters studied included endothelial cell density and hexagonality. The data obtained were expressed as mean ± SD. and by using one-way analysis of variance to compare the data among age groups. Differences were considered statistically significant if the P value was less than 0.05.

RESULTS

No changes were noted in the slit lamp examination. With the specular microscope it was possible to analyse, capture images, and quantify the cell density and hexagonality of the corneal endothelium. In all the analysed images a regular pattern of size and shape of the endothelial cells was observed throughout the entire cornea (Figure. 1). The mean cell density for GI was 2307 cells per mm^2, for GII was 1895 cells per mm^2 , and for GIII was 1818 cells per mm^2 (Table. 1). For GI, the mean cell density was 2336±367.99 cells per mm^2 for the right eye and 2278.33±294.15 cells per mm^2 for the left eye. For

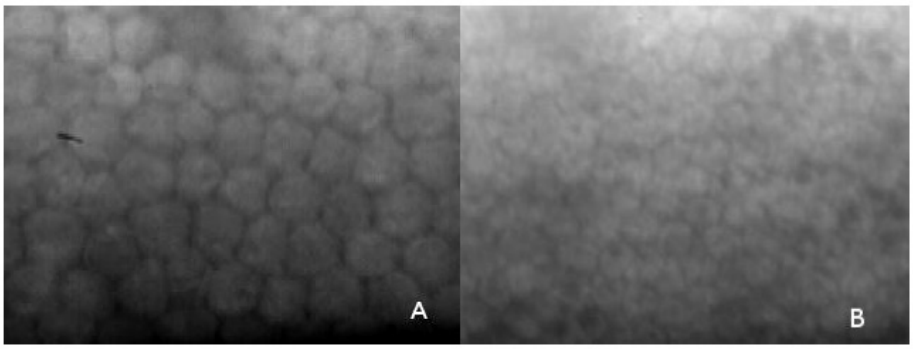

Figure 1 - Specular micrographs of the normal corneal endothelium of a rabbit. A. Normal corneal endothelium of a rabbit from G III. B. Normal corneal endothelium of a rabbit from G I.

Table 1 - Mean endothelial cell density (cell mm^{-2}) and pleomorphism in rabbits evaluated through specular microscopy.

Group	I	II	III
Mean endothelial density	2307	1895	1818
Pleomorphism	74.33	71.83	64.02

GII, the mean cell density was 1875.17±164.26 cells per mm^2 for the right eye and 1914.33±190.50 cells per mm^2 for the left eye. For GIII, the mean cell density was 1865.83±283.29 cells per mm^2 for the right eye and 1771.50±215.84 cells per mm^2 for the left eye. Cell density decreased significantly with age ($P<0.001$) among the three groups.

Pleomorphism in the rabbits of GI was 74.33±10.08. In the rabbits of GII it was 71.83±11.38. In the rabbits of GIII it was 64.02±28.80. Significant differences ($P<0.001$) were evident between GI and GIII and GII and GIII but not between Groups I and II. The data obtained did not differ significantly between the right and the left eye from the same rabbit.

DISCUSSION

Specular microscopy, optical microscopy, and scanning electron microscopy (SEM) are among the most widely used methods for corneal endothelium analysis (MORITA et al., 1994; PIGATTO et al., 2009; ALBUQUERQUE et al., 2015; COYO et al., 2016; FAGANELLO et al., 2016). There is consensus that the preparation of corneas for studies using SEM causes cell retraction by reducing the original cell area and increasing the endothelial cell density (VIRTANEN et al., 1984; FAGANELLO et al., 2016).

Nevertheless, SEM has been used to study the ultrastructure of endothelial cells of different species of animals (PIGATTO et al., 2004; PIGATTO et al., 2005a; PIGATTO et al., 2005b; PIGATTO et al., 2009; TAMAYO-ARANGO et al., 2009). Due to the fact that there is no cellular retraction and it can be used in animals and living humans, specular microscopy is considered the gold standard technique for assessing the corneal endothelium in people and animals (MORITA, 1995; ABIB & BARRETO, 2001; PIGATTO et al., 2006; PIGATTO et al., 2008; FRANZEN et al., 2010; BERCHT et al., 2015; ALBUQUERQUE et al., 2015; COYO et al., 2016). Specular microscopy has also been employed in the evaluation of dogs undergoing cataract removal surgery (NAGATSUYU et al., 2014). Specular microscopy is a widely used tool in many researches, and been demonstrated to be extremely reliable, and reproducible. In healthy rabbits observations of the corneal endothelium employing a specular microscope have already been performed (SAILSTAD & PEIFFER, 1981; MORITA, 1995). In previous studies, rabbits underwent general anaesthesia prior to specular microscopy examination (SAILSTAD & PEIFFER, 1981; MORITA, 1995). However, in the present study we made the choice to use the eyes of animals. This methodology using the eyes of animals that have been sacrificed has been used successfully in previous studies (PIGATTO et al., 2006; PIGATTO et al., 2008; FRANZEN et al., 2010; ALBUQUERQUE et al., 2015; COYO et al., 2016). It is well established that the endothelial structure is preserved for up to 6 hours after death (FRANZEN et al., 2010; ALBUQUERQUE et al., 2015). In the current study, the maintenance of the eyes in a humid chamber allowed the examinations without interfering with the transparency of the cornea. The use of eyes from abattoirs or breeders avoids animals being anaesthetized only to perform specular microscopy. Moreover, results obtained with this methodology can be extrapolated and compared to values obtained from living animals.

Rabbits have been widely used as an experimental model for ophthalmic research (ATILLA et al., 2003; MENCUCCI et al., 2005; ARI et al., 2015). The goal of the present study was to evaluate the cell density and percentage of hexagonal cells because these are the most reliable parameters for evaluating the integrity of the corneal endothelium (ABIB & BARRETO, 2001; PIGATTO et al., 2006; NAGATSUYU et al., 2014). SAILSTAD and PEIFFER (1981) observed the corneal endothelium of 14 young adult rabbits using a specular microscope. The authors reported an average density of 2998 cells per square millimeter. MORITA (1995), studying corneal endothelial cell of rabbit eyes by specular microscopy reported an endothelial cell density between 2180 and 3460 cells per mm^2. In the present study, the endothelial cell density varied between 2336 and 1771.50 cells per mm^2. In other studies, scanning electron microscopy was used to quantify the endothelial density in rabbits (DOUGHTY, 1998; PIGATTO et al., 2005a). However, due to the cellular retraction caused by the preparation for scanning electron microscopy and the increase in the number of cells per square millimeter, they cannot be compared with the values obtained with a specular microscope. It is very well established

in many species that a decrease in endothelial density occurs with aging (MORITA et al., 1994; FRANZEN et al., 2010; ALBUQUERQUE et al., 2015; BERCHT et al., 2015; COYO et al., 2016). Results of this study regarding the mean cell density in different age groups were similar to the results obtained for other species of animals with a mean decrease in endothelial cell density with increasing age.

Regarding the endothelial morphology in rabbits, there are no studies evaluating the effect of aging on corneal endothelial hexagonality in rabbits. MORITA (1995) examined rabbits aged between 6 months and 15 months with a specular microscope and found cells with five and six sides. PIGATTO et al. (2005a) evaluated the morphology of 3-month-old rabbits using SEM with a focus on polygonality of the endothelium. Most cells were hexagonal (75%) in shape, with pentagonal (14%) and heptagonal (11%) cells constituting the greater portion of the remaining corneal endothelium. Some studies evaluating animals of a unique age group have already been conducted and concluded that most cells have six sides (PIGATTO et al., 2004; PIGATTO et al., 2005a; PIGATTO et al., 2006; PIGATTO et al., 2008;IGATTO et al., 2009; TAMAYO-ARANGO et al., 2009; FRANZEN et al., 2010; ALBUQUERQUE et al., 2015; COYO et al., 2016). In humans with a healthy cornea, more than 60% of the corneal endothelium cells are six-sided (McCAREY et al., 2008). A decrease in the number of endothelial cells with six sides commonly occurs with aging (ALBUQUERQUE et al., 2015; COYO et al., 2016). In the present study, it was observed that with the aging of the rabbits there was a decrease in the number of hexagonal-shaped cells. With regard to the parameters evaluated, no statistical differences were observed between both eyes of the same rabbit. Previous studies reported the absence of differences regarding the endothelial parameters obtained between healthy eyes (MORITA et al., 1994; PIGATTO et al., 2006; PIGATTO et al., 2008; FRANZEN et al., 2010; ALBUQUEQUE et al., 2015; BERCHT et al., 2015). These results obtained in the present study were consistent with those reported in rabbits were the density of corneal endothelial cells is similar in the left and the right eye and in females and males.

CONCLUSION

Corneal endothelium of rabbits suffers changes due to advancing age both in terms of cell density and morphology. The data obtained in this study may serve as a reference for further studies.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This research was approved by the Research Committee of the Faculty of Veterinary of the Universidade Federal do Rio Grande do Sul (UFRGS), and followed the ethical norms of the Association for Research in Vision and Ophthalmology (ARVO).

REFERENCES

ABIB, F.C., BARRETO, J.R. Behavior of corneal endothelial density over a lifetime. **Journal of Cataract and Refractive Surgery**, v.27, n.10, p.1574-78, 2001. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/11687354>. Accessed: Sept. 21, 2015. doi: 10.1016/S0886-3350(01)00925-7.

ALBUQUERQUE, L. et al. Analysis of the corneal endothelium in eyes of chickens using contact specular microscopy. **Semina**: **Ciências Agrárias**, Londrina, v.36, n.6, p.4199-4206, 2015. Available from: <http://www.uel.br/revistas/uel/index.php/semagrarias/article/viewFile/19497/17540>. Accessed: Mar. 07, 2016. doi: 10.5433/1679-0359.2015v36n6Supl2p4199.

ANDREW, S. et al. Density of corneal endothelial cells and corneal thickness in eyes of euthanatized horses. **American Journal of Veterinary Research**, v.62, n.4, p.479-482, 2001. Available from: <http://avmajournals.avma.org/doi/abs/10.2460/ajvr.2001.62.479?journalCode=ajvr>. Accessed: Mar. 10, 2014. doi: 10.2460/ajvr.2001.62.479.

ARI, S. et al. Evaluation of intracameral injection of ranibizumab and bevacizumab on the corneal endothelium by scanning electron microscopy. **Journal of Ocular Pharmacology and Therapeutics**, v.31, n.2, p.100-105, 2015. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/?term=Evaluation+of+intracameral+injection+of+ranibizumab+and+bevacizumab+on+the+corneal+endothelium+by+scanning+electron+microscopy>. Accessed: Sept. 15, 2015. doi: 10.1089/jop.2014.0005.

ATILLA, H. et al. Effects of intracameral lidocaine on ocular tissues. **Clinical and Experimental Ophthalmology**, v.31, n.1, p.73-77, 2003. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/12580899>. Accessed: Sept. 16, 2015. doi: 10.1046/j.1442-9071.2003.00604.x.

BERCHT, B.S. et al. Specular microscopy to determine corneal endothelial cell morphology and morphometry in chinchilas (*Chinchilla lanigera*) in vivo. **Veterinary Ophthalmology**, v.18, n.1, p.137-142, 2015. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25469785>. Accessed: June 11, 2016. doi: 10.1111/vop.12236.

COYO, N. et al. cEffects of age and breed on corneal thickness, density, and morphology of corneal endothelial cells in enucleated sheep eyes.c **Veterinary Ophthalmology**, v.19, n.5, p.372-375, 2016. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/26338229>. Accessed: Mar. 15, 2016. doi: 10.1111/vop.12308.

DOUGHTY, M.J. Changes in cell surface primary cilia and microvilli concurrent with measurements of fluid flow across the rabbit corneal endothelium ex vivo. **Tissue and Cell**, v.30, n.6, p.634-643, 1998. Available from: <http://www.sciencedirect.com/science/article/pii/S0040816698800827>. Accessed: Oct. 05, 2013.

FAGANELLO, C.S. et al. Morphology of endothelial cells from different regions of the equine cornea. **Ciência Rural**, v.46, n.12, p.2223-2228, 2016. Available from: <http://dx.doi.org/10.1590/0103-8478cr20160216>. Accessed: Nov. 15, 2016. doi: 10.5433/1679-0359.2015v36n6Supl2p4199.

FRANZEN, A.A. et al. Use of specular microscopy to determine corneal endothelial cell morphology and morphometry in enucleated cat eyes. **Veterinary Ophthalmology**, v.13, n.4, p.222-226, 2010. Available from: <http://hdl.handle.net/11449/41931>. Accessed: Oct. 09, 2013. doi: 10.1111/j.1463-5224.2010.00787.x.

McCAREY, B.E. et al. Review of corneal endothelial specular microscopy for FDA clinical trials of refractive procedures, surgical devices and new intraocular drugs and solutions. **Cornea**, v.27, n.1, p.1-16, 2008. Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3062434/>. Accessed: Sept. 13, 2013. doi: 10.1097/ICO.0b013e31815892da.

MENCUCCI, R. et al. Ultrasound thermal damage to rabbit corneas after simulated phacoemulsification. **Journal of Cataract Refractive surgery**, v.31, n.11 p.2180-2186, 2005. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/16412936>. Accessed: Sept. 10, 2016. doi: 10.1016/j.jcrs.2005.04.043.

MORITA, H. Specular microscopy of corneal endothelial cells in rabbits. **Journal of Veterinary Medicine and Science**, v.57, n.2, p.273-277, 1995. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/7492645>. Accessed: Sept. 15, 2013.

MORITA, H. Specular microscopy of corneal endothelial cells in cynomolgus monkeys. **Journal of Veterinary Medicine and Science**, v.56, n.4, p.763-764, 1994. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/7999905>. Accessed: Sept. 15, 2015. doi: 10.1292/jvms.56.763.

NAGATSUYU, C.E. et al. Non-contact specular microscopy in aphakic and pseudophakic dogs. **Ciência Rural**, v.44, n.4, p.682-687, 2014. Available from: <http://dx.doi.org/10.1590/S0103-84782014000400018>. Accessed: Nov. 10, 2016. doi: 10.1590/S0103-84782014000400018.

PIGATTO, J.A.T. et al. Morphometric analysis of the corneal endothelium of yacare caiman (*Caiman yacare*) using scanning electron microscopy. **Veterinary Ophthalmology**, v.7, n.3, p.205-208, 2004. Available from: <http://www3.interscience.wiley.com/journal/118811881/abstract/>. Accessed: Sept. 11, 2013. doi: 10.1111/j.1463-5224.2004.04025.x.

PIGATTO, J.A.T. et al. Análise morfométrica do endotélio corneano de coelhos à microscopia eletrônica de varredura. **Acta Scientiae Veterinariae**, v.33, n.1, p.33-39, 2005a. Available from: <http://www.redalyc.org/pdf/2890/289021867004.pdf>. Accessed: Oct. 10, 2013.

PIGATTO, J.A.T. et al. Corneal endothelium of the magellanic penguin (*Spheniscus magellanicus*) by scanning electron microscopy. **Journal of Zoo and Wildlife Medicine**, v.36, p.702-705, 2005b. Available from: <http://www.bioone.org/doi/abs/10.1638/05017.1>. Accessed: Oct. 10, 2013. doi: 10.1638/05017.1.

PIGATTO, J.A.T. et al. Density of corneal endothelial cells in eyes of dogs using specular microscopy. **Brazilian Journal of Veterinary Research and Animal Science**, v.43, n.4, p.476-480, 2006. Available from: <http://www.uel.br/revistas/uel/index.php/semagrarias/article/viewFile/19497/17540>. Accessed: Mar. 14, 2016. doi: 10.5433/1679-0359.

PIGATTO, J.A.T. et al. Morphological analysis of the corneal endothelium in eyes of dogs using specular microscopy. **Pesquisa Veterinária Brasileira**, v.28, n.9, p.427-430, 2008. Available from: <http://www.scielo.br/scielo.php?pid=S0100-736X2008000900006&script=sci_arttext>. Accessed: Sept. 09, 2016. doi: 10.1590/S0100-736X2008000900006.

PIGATTO, J.A.T. et al. Scanning electron microscopy of the corneal endothelium of ostrich. **Ciência Rural**, v.39, n.3, p.926-929, 2009. Available from: <http://dx.doi.org/10.1590/S0103-84782009005000001>. Accessed: Oct. 20, 2013. doi: 10.1590/S0103-84782009005000001.

SAILSTAD, D.M.; PEIFFER, R.L. Specular microscopic observations of the corneal endothelium in the normal rabbit. **Laboratory Animals**, v.15, n.4, p.393-395, 1981. Available from: <http://lan.sagepub.com/content/15/4/393.full.pdf>. Accessed: Feb. 02, 2016. doi: 10.1258/002367781780952924.

TAMAYO-ARANGO, L.J. et al. Ultrastructural morphology and morphometry of the normal corneal endothelium of adult crossbred pig. **Ciência Rural**, v.39, n.1, p.117-122, 2009. Available from: <http://dx.doi.org/10.1590/S0103-84782009000100018>. Accessed: Mar. 06, 2014. doi: 10.1590/S0103-84782009000100018.

TERZARIOL, M. et al. Effects of intracameral brilliant blue on the corneal endothelium of swine: in vitro study. **Pesquisa Veterinária Brasileira**, v.36, n.8, p.775-780, 2016. Available from: <http://dx.doi.org/10.1590/S0100-736X2016000800016>. Accessed: Nov. 15, 2016. doi: 10.1590/S0100-736X2016000800016.

VIRTANEN, J. et al. The effect of fixation on corneal endothelial cell dimensions and morphology in scanning electron microscopy. **Acta Ophthalmologica**, v.62, n.4, p.577-585, 1984. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/6435388>. Accessed: Sept. 11, 2013. doi: 10.1111/j.1755-3768.1984.tb03970.x.