



## Diminished angiogenesis in the cornea of mice with heterologous deletion of Connexin 43 gene (*Gja1*).

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### Abstract

Angiogenesis is involved in several physiological and pathological processes, and the proliferation of endothelial cells is a central event in the generation of new blood vessels. Gap junctions (GJ) are membrane structures that communicate adjacent cells, contribute to tissue homeostasis, and are important to the control of cell proliferation. Connexins (Cx) are the proteins that form gap junctions. Endothelial cells may express Cx43, Cx37 and Cx40. In this study, we analyzed the effect of the heterologous deletion of the *Gja1* (Cx43 gene) on the development of newly formed blood vessels (NFBV) in the mouse cornea. Heterozygous (Cx43<sup>+/-</sup>) and wild-type (Cx43<sup>+/+</sup>) mice were submitted to the silver crystal corneal cauterization model. Two parameters were quantified by image analysis 2 or 6 days after cauterization: NFBV density and length. At days 2 and 6 after corneal cauterization, Cx43<sup>+/-</sup> mice showed smaller density of NFBV as compared to Cx43<sup>+/+</sup> mice. Therefore, deletion of one *Gja1* allele (connexin-43 gene) may lead to impaired cell-cell communication in endothelial cells, diminishing angiogenesis after cauterization of the mouse cornea.

**Key Words:** gap junctions, connexin, *Gja1*, angiogenesis, endothelial cell, cornea

**Abbreviations:** GJ: gap junctions, Connexin 43: Cx43; Connexin 37: Cx37, Connexin 40: Cx40; Cx43<sup>+/+</sup> wild type mice; Cx43<sup>+/-</sup> heterozygous mice; NFBV: newly formed blood vessels, *Gja1*: gap junction alpha 1

### Introduction

Angiogenesis is involved in several physiological and pathological processes, being an essential event during reproduction, development, and wound repair. Control of angiogenesis may be desirable in diseases that are driven by persistent unregulated angiogenesis, such as arthritis, diabetic retinopathy, tumor growth, and cancer metastasis (10, 11). Therefore, it is highly important to understand the events that can influence endothelial cell growth to form new blood vessels.

Endothelial growth is the central event during formation of new blood vessels (10). One of the factors that can influence cell proliferation is the ability to communicate with adjacent cells (5.). Although particularly complex (28), understanding molecular

pathways of crosstalk between endothelial cells is crucial for the comprehension of angiogenesis dynamics.

Several mechanisms of cell communication have been widely described, and gap junction intercellular communication (GJIC) is certainly the most direct one. Gap junctions (GJ) are cell membrane specializations that permit direct communication of adjacent cells. Each GJ is composed by 2 hemichannels (connexons) from each adjacent cell. Each connexon is a hexameric structure of connexin (Cx) (12, 30). The connexin family, which number at least is 20 highly conserved proteins are expressed in different tissue in a specific manner (20). There are, at least 20 Small hydrophilic molecules and ions (<1-2 kDa) are constantly exchanged between neighboring cells (23, 33), playing important roles in the synchronized

vasoactive responses, growth responses and second-messenger signaling in the vascular wall (2).

Connexins (Cxs) are the main proteins that form gap junctions (12). Several tissues and individual cells commonly express more than one type of connexin. Differential expression of Cxs can contribute to functional differentiation in the vascular network (34). The expression of Cx43, Cx37 and Cx40 in endothelial cells has been previously described, depending on the localization of blood vessels (13, 14, 21).

The corneal model of angiogenesis has been used by several authors (4, 25), and represents one of the best *in vivo* models to study angiogenesis. The cornea is devoid of pre-existing vascularization, and therefore any vessels found there after cauterization or stimulation by angiogenic factors are newly formed blood vessels (NFBV) (1).

The Cx 43 is the most widely expressed connexin, and its expression is also found in the corneal endothelium (31). It has been shown the participation of connexin 43 during wound healing in the rabbit corneal epithelium (16). The role of Cx 43 during wound healing is also demonstrated in other tissues like the tongue (24) and epidermis (3). At 3 hours after scrape injury, the expression of Cx 43 in rat corneal endothelium decreases around the wound edge (17).

Genetically manipulated mice represent a new set of tools in science, contributing to the investigation on the roles of genes in physiological or pathological processes by its deletion, replacement, or over-expression. Connexin 43 knockout mice have been used to investigate the roles of this protein in physiology and disease. However, only heterozygous mice can be used, since the homozygous knockout mice die shortly after birth. In this study, we employed the classical model of corneal cauterization in Cx43 heterozygous knockout mice in order to study the role of *Gjal* in angiogenesis.

## Materials and methods

### Animals

Cx43<sup>+/-</sup> mice were obtained from the International Agency for Research on Cancer (IARC, Lyon, France). Mice were generated by replacing the exon-2 of the Cx43 gene by the neomycin resistance gene (22). The mice were produced originally in the C57BL/6 strain, and the background was later changed to CD1 by serial breeding at IARC animal facility. Due to the perinatal lethality of Cx43-knockout mice, only heterozygous (Cx43<sup>+/-</sup>) and wild type (Cx43<sup>+/+</sup>) mice were used in this study. These mice were weaned at the age of 4 weeks and housed under controlled conditions (22°C ± 2°C; 65 ± 15% relative humidity, air exchange rate 15 times/h, 12h-12h light-dark cycle), in filter top cages. The animals received a standard pellet diet (Purina Lab Chow, Brazil) and tap water *ad libitum* during all the study.

### Genotyping of mice by Polymerase Chain Reaction (PCR)

DNA was harvested from the tail of each mouse and was analyzed by PCR as previously described (32). The reaction products were loaded on an agarose gel (1.5%, diluted in TBS buffer). Typical bands of amplified *Gjal* gene (520 bp) and *neo* gene (294 bp) were obtained (Figure 1). Once the mice were genotyped, they were separated into the different experimental groups used in this study.

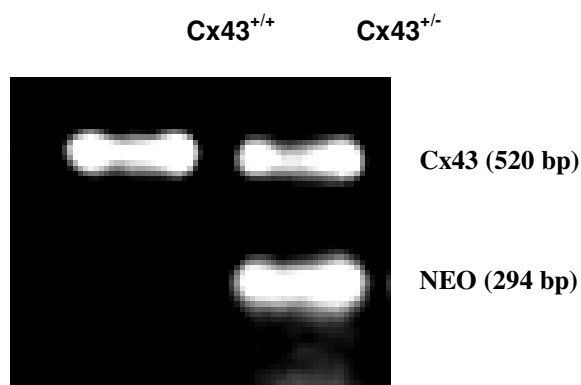


Figure 1. Genotyping of Cx43<sup>+/+</sup> and Cx43<sup>+/-</sup> mice by PCR.

### Chemical cauterization of the mouse cornea

Corneal cauterization for the induction of angiogenesis was performed as described elsewhere (7). Eight week-old male or female mice were anesthetized by intraperitoneal injection of 60 mg of pentobarbital (Hypnol® 3%), and a few drops of topical anesthetic (Visonest® proxymetacaine) were administered to each eye. Both corneas were cauterized with a sharp silver nitrate crystal, which was applied to the center of the cornea during 10 seconds and the eyes were rinsed with distilled water. For post-operative analgesia 0.1 mg of buprenorphine (Temgesic®, Shering-Plough) was given subcutaneously at 12-hour intervals, to a total of 3 administrations per mouse. This procedure was approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil (Process number 322/2003).

### Morphometry of NFBV in the mouse cornea

NFBV were quantified in two different time points: 2 days (26 males and 28 females) or 6 days (28 males and 24 females) after corneal cauterization.

On the second or sixth day post-cauterization, the animals were deeply anesthetized and received 500 I.U. of heparin to avoid intravascular clotting; 10 mL of PBS were injected intravenously to clear the remaining blood from the vessels, followed by 10 mL of colloidal carbon (India ink, Faber Castell®, black) to fill vascular beds. After euthanasia, eyeballs were excised and fixed in 10% formalin for 48 hours, after which they were dehydrated in crescent ethanol grades (50-100%), cleared in benzol and stored in Spaltholz

solution (5:3 methyl salicylate:benzyl benzoate). The corneas were removed and sectioned in two similar halves, mounted and extended on glass slides for 24 hours, and coverslipped with synthetic resin (Permount®). Samples were observed under an E-800 Nikon microscope, to which a Nikon Cool Pix digital camera and the Image Pro-Plus analysis system were attached.

The density of NFBV was quantified to evaluate the percentage of the cornea taken by new vessels. The image of the cornea was captured at 40X magnification. Regions of 638 mm<sup>2</sup> immediately above the limbus were established, and the proportion of black structures (blood vessels filled with colloidal carbon) was quantified. The absolute lengths achieved by NFBV, as well as the distance from the limbus to the edge of the cauterization lesion, were measured. A percentage value using raw data of the parameters described above (length and limbus-to-lesion distance) was determined and accepted as the adjusted length of NFBV.

#### Statistical analysis

Statistical comparison of NFBV data was performed by ANOVA. Values of P<0.05 were considered significant throughout the study.

#### Results

##### Quantification of NFBV 2 and 6 days after cornea cauterization

The density of NFBV in cornea was significantly lower in Cx43<sup>+/-</sup> mice in comparison to Cx43<sup>+/+</sup> mice at days 2 or 6 after cauterization (Table 1). This was verified in both male and female mice. There were no differences in the length of NFBV in mice of different genotypes at day 2 or day 6 after cornea cauterization.

Figures 2 and 3 show NFBV at days 2 or 6 after corneal cauterization in mice.

**Table 1.** Density and length of NFBV after 2 or 6 days of corneal cauterization. Male or female Cx43 heterozygous (Cx43<sup>+/-</sup>) or wild-type (Cx43<sup>+/+</sup>) mice were used. Values are expressed as means  $\pm$  standard deviation. \*ANOVA (p<0.05).

		Day 2 after cornea cauterization		Day 6 after cornea cauterization	
		Density of NFBV (number of animals)	Length of NFBV (number of animals)	Density of NFBV (number of animals)	Length of NFBV (number of animals)
Males	Cx43 <sup>+/+</sup>	21.53 $\pm$ 6.18 (n=14)	88.35 $\pm$ 19.28 (n=14)	13.43 $\pm$ 4.61 (n=15)	75.63 $\pm$ 20.51 (n=15)
	Cx43 <sup>+/-</sup>	10.04* $\pm$ 2.42 (n=12)	67.86 $\pm$ 19.57 (n=12)	7.88* $\pm$ 4.04 (n=13)	80.24 $\pm$ 20.95 (n=13)
Females	Cx43 <sup>+/+</sup>	21.98 $\pm$ 3.88 (n=15)	87.80 $\pm$ 2.24 (n=15)	17.08 $\pm$ 3.88 (n=11)	86.12 $\pm$ 15.26 (n=11)
	Cx43 <sup>+/-</sup>	12.63* $\pm$ 3.32 (n=13)	85.09 $\pm$ 19.25 (n=13)	11.21* $\pm$ 3.67 (n=12)	81.77 $\pm$ 19.96 (n=12)

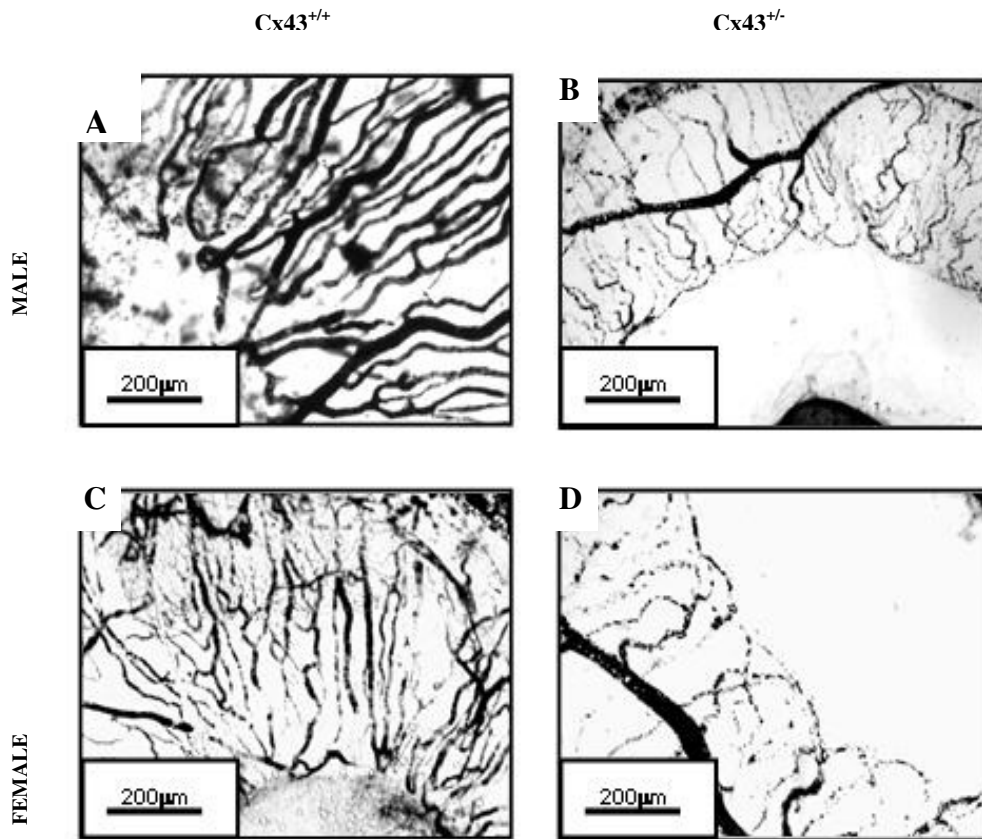


Figure 2. NFBV in the mouse cornea at day 2 post-cauterization. The India ink area (black) corresponds to NFBV. A: male  $Cx43^{+/+}$ ; B: male  $Cx43^{+/-}$ ; C: female  $Cx43^{+/+}$ ; D: female  $Cx43^{+/-}$ . Arrowheads show the cauterization site in mice cornea.

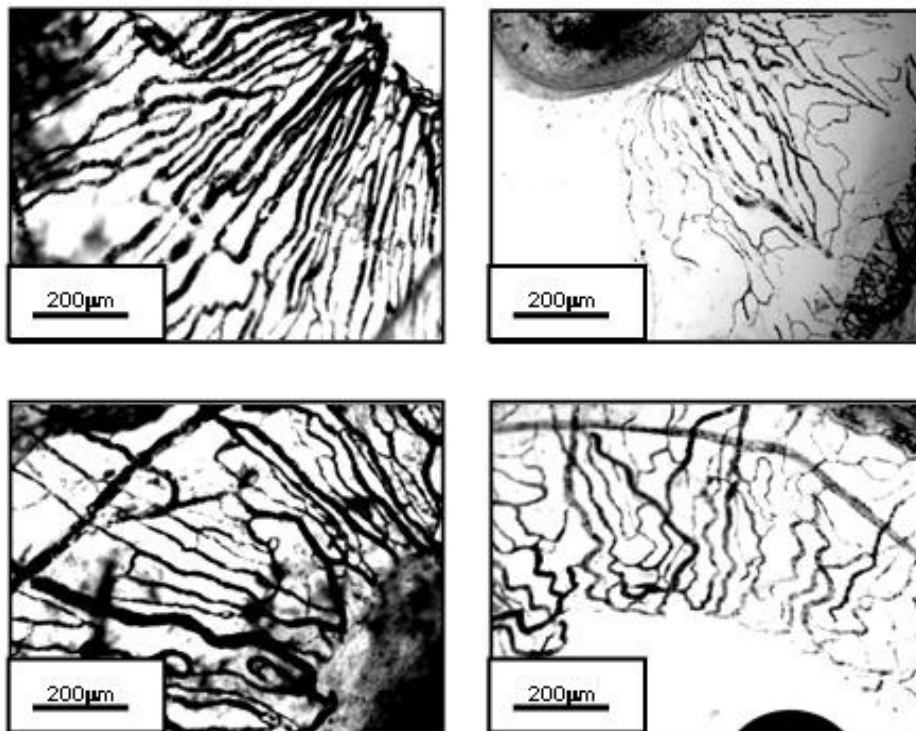


Figure 3. NFBV in the mouse cornea at day 6 post-cauterization. The India ink area (black) corresponds to new vessels. A: male  $Cx43^{+/+}$ ; B: male  $Cx43^{+/-}$ ; C: female  $Cx43^{+/+}$ ; D: female  $Cx43^{+/-}$ . Arrowheads show the cauterization site in mice cornea.

## Discussion

Understanding the mechanisms involved in angiogenesis is important to its control (9). The aim of the present work was to contribute to the detection of factors that underlie endothelial cell proliferation during the generation of new blood vessels.

Cx43 knockout mice have been used as an *in vivo* model to evaluate the role of Cx43 on the development of NFBV. Connexin 43 heterozygous mice breed and develop normally. However, homozygous Cx43 (Cx43<sup>-/-</sup>) mice die shortly after birth. It was recently described that Cx43-deficient mice present altered coronary vasculogenesis (29). Our results supply details on the roles of connexins, particularly Cx43, in angiogenesis.

In the corneal cauterization model, the chemical injury is the stimulus for endothelial cell proliferation. NFBV vessels drive straightforward to the edge of the lesion. Endothelial cells, derived from sprouts of limbic blood vessels, grow continuously after cornea injury. Cell growth apparently peaks at day 2 after cornea cauterization, and blood vessels growth lasts about 5 days (25). For this reason, we chose days 2 and 6 to evaluate the growth of NFBV in mouse cornea. NFBV density was smaller in both male and female Cx43 heterozygous knockouts at days 2 and 6 post-cauterization. However, the NFBV length was not different in the 2 time points investigated.

The maintenance of gap junction intercellular communication capacity is crucial for maintaining tissue homeostasis. An inverse relationship between GJIC and cell proliferation has been reported. According to Loewenstein, 1981 (15) and Trosko and Ruch, 2002, (27) a communication-deficient cell could not exchange inhibitory nor stimulatory factors, being unable to obey the homeostatic mechanisms present in its respective tissue.

Therefore, as a consequence, a communication-deficient cell would present increased cell proliferation. Based on this, we would rather believe that Cx43 deficient endothelial cells could grow faster than their normal counterparts. Unexpectedly, after chemical injury, leading to corneal cauterization, blood vessels grew significantly slowly in heterozygous knockout mice. Recently, Nakano and cols, 2008 (17), studied the role of Cx 43 in rat corneal healing. After the injury, the expression of Cx 43 in endothelium drastically changes, and the Cx43Knockdown treatment enhances wound healing. A single application of Cx 43 AS-ODN or siRNA into the anterior chamber simultaneously, accelerated wound closure and increase the number of Ki67-positive proliferation cell in the corneal endothelium.

It is widely accepted that neoplastic cells present fewer gap junctions and display a lower gap junction communication capacity. According to Trosko and Ruch, 1998, (27) gap junction size and number, connexin expression and cell-cell coupling (GJIC) have been studied in many neoplastic cells using structural and biochemical parameters, and by

the introduction of fluorescent or radioactive tracers, with the determination of tracer transfer into adjacent cells. The vast majority of neoplastic cells have fewer and smaller gap junctions, express less connexins, and have reduced GJIC compared to their non-neoplastic counterparts.

Drugs that avoid endothelial cells proliferation and communication could be an important strategy of cancer treatment by controlling tumor growth and metastasis. Chou and others (2007) (6) showed reduction of Cx43 expression and inhibition of gap-junction communication in human aortic endothelial cells after treatment with arsenic trioxide (As<sub>2</sub>O<sub>3</sub>), anti-cancer drug. These result shows that Cx43 inhibits could be important way to reduce tumor growth and metastasis.

Excessive cell proliferation in neoplastic tissue is due to permanent alterations in growth-control genes. Gap junctions could contribute to the accumulation of these growth-stimulatory proteins that would result in higher cell proliferation. Apparently, there is a paradox between neoplasia and hyperplasia, as seen here in corneal angiogenesis. In this situation, there is also an increase in the expression of growth control genes; however, it is noteworthy that this stimulation is transitory, being suppressed by the end of the hyperplastic phase of this process. We suggest that the relationship between gap junction, connexins and cell proliferation can be different in neoplastic and non-neoplastic processes. Gap junctions play a role not only in growth suppression, but also in coordinating cell growth, i.e. both positively and negatively to achieve a normal growth rate (8 ).

In conclusion, in this study we show, for the first time, that Cx43 plays a role in the generation of new blood vessels in mouse cornea, and *Gjal* deficiency promotes the reduction of angiogenesis.

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