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Compensatory kidney hypertrophy/hyperplasia after nephrectomy in mice: alterations of connexin 43 (Cx43) phosphorylated isoforms

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Abstract

Compensatory kidney hypertrophy/hyperplasia leads to several changes in kidney structure and function, as increased glomeruli filtration. The aim of this study was to evaluate connexin 43 in remnant mouse kidneys after unilateral nephrectomy. The right kidney was surgically removed from BALB/c mice. Groups were euthanized at 24, 48 and 72 hours, and at 7 and 30 days. Kidney sections of the reminiscent kidneys were stained with Periodic Acid/Schiff and additional slides were submitted to BrdU and Cx43 immunohistochemistry. The results demonstrated an increase in kidney weight as early as 24 hours through 30 post-nephrectomy. In addition, BrdU positive epithelial cells increased at 24 and 48 hours post-nephrectomy. Cx43 was detected in the cytoplasm and membrane of epithelial cells and vasculature. Taking into consideration the quantity, intensity and localization of Cx43 immunostaining pattern, we observed that nephrectomized mice presented lower Cx43 expression and a cytoplasmic localization after 24 hours, peaking in 48 hours. Furthermore, western blot revealed that during the first 24 and 48 hours after nephrectomy, P0 (unphosphorylated) and P1 (phosphorylated) Cx43 disappeared, and the products of Cx43 degradation were reduced. On the other hand, after 72 hours the P0 and P1 state reappeared and the amount of degraded peptides also increased. Seven and thirty days after nephrectomy, a higher intensity of P0 and P1 state and a lower P2 (hyperphosphorylated) band were observed. In conclusion, our results suggest that Cx43 phosphorylation results in the retention of Cx43 in cytoplasm and its increased degradation during compensatory renal hyperplasia/hypertrophy.

Keywords: kidney, nephrectomy, hyperplasia/hypertrophy, connexin, gap junction

Introduction

Compensatory kidney hypertrophy was described as a whole set of changes in the structure and function of the kidney that follows the reduction of its mass (15). This process is frequently regarded in humans and animals after nephrectomy.

Cortical, internal and external medullary areas are the major regions with intense mitotic activity observed immediately after nephrectomy (21). Therefore, the higher

mitotic indexes after nephrectomy are observed 48 hours in proximal tubular cells and in the distal tubular cells. Ascendant loop and collector tubules showed mostly two major cell proliferation peaks, at 40 hours and at 3 and 4 days after intervention (6, 25).

Renal hypertrophic cells are frequently arrested at G1 phase, and show alterations in enzyme activity, ions exchange, and altered gene expression (1,15). These alterations promote a non-selective renal cell growth. In addition, the compensatory renal growth was recently related to a reduced gap junction intercellular communication (12).

Gap junction intercellular communication (GJIC) has also been related to liver regeneration after partial hepatectomy (19) and was thought to regulate cell growth (13). These junctions are formed by connexons on contiguous cell membranes, each of which is a hexamer of connexin (Cx) proteins. Connexins are a family composed of at least twenty-one members (26). In kidney tissue Cx26, Cx30.3, Cx31, Cx37, Cx40, Cx43, Cx45 and Cx46 are expressed. Due to the complex heterogeneity of renal histology, the localization of all these connexins is unknown (7). Nevertheless, it is known that Cx43 is distributed by whole adult kidney (23). Moreover, Cx43 is detected in proximal and distal tubules, glomerulus, internal medullar collecting duct, and pelvic epithelia, and is positively relevant in arteries, capillaries and in juxtaglomerular cells (2, 3).

The regulation of gap junction assembly and turnover are important factors in the control of intercellular communication. Evidence for acute regulation of connexins is observed in renal cell carcinoma, where a loss of GJIC function is largely associated with changes in the phosphorylation levels and Cx43 localization (20,24). In addition, Cx43 expression is also related to abnormal growth during glomerulonephritis and it was also implicated in distal tubules inflammatory process (8). In this study, morphology, cell proliferation and Cx43 isoforms by SDS-PAGE were characterized during renal hyperplasia/hypertrophy post-nephrectomy in mice.

Material and Methods

Animals. BALB/c adult male mice (25/30 g) were obtained from the Faculty of Veterinary Medicine and Animal Sciences, University of São Paulo, facility and housed under controlled environment (22□C □ 2□C; 65 □ 15% relative humidity; air exchange rate 15 times/h, 12h-12h light-dark cycle). All animals received a pelleted standard diet (Purina Lab Chow, Curitiba, Brazil) and tap water ad libitum during the study. All procedures using animals were performed following "Principles of laboratory animal care" (NIH publication No. 85-23, revised in 1985) and were reviewed and approved by the Bioethics Committee of the FMVZ-USP (protocol number 71/2002).

Nephrectomy. Seventy mice were anesthetized (ether inhalation) and the right kidney was accessed, renal hilus was ligated using a 4-0 silk suture and extirpation was performed. Sham operations (control) consisted of a skin incision and sham-operated left kidneys were used as controls.

Experimental protocol. The animals were euthanized 24, 48 and 72 hours and at 7 and 30 days after nephrectomy. One hour before euthanasia all mice received a BrdU (Sigma, 10 mg/kg body weight) intra-peritoneal injection. In addition, all mice and their respective kidneys were weighed and representative slices were fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid).

Morphometry and BrdU quantification. To perform kidney morphometrical analysis, representative slides were stained with periodic Schiff acid (PAS) and were quantified in a microscope (Nikon Eclipse-800) and an analysis system (Image Pro-Plus version 4.5, Media Cybernetics). In each kidney section, 1000 cells were counted, distinguishing its histological localization. To quantify BrdU positive cells, we used an immunohistochemistry protocol, with an anti-BrdU antibody and a secondary byotinilated mouse anti-immunoglobulin antibody (1:1000) followed by streptavidin-biotin-peroxidase complex (duet kit, DAKO, California, EUA) according to proceeding of the manufacturer. Approximately 1000 nuclei were counted in tubular epithelial cells in random fields (at 20x magnification).

Immunohistochemical detection of Cx43. Kidneys obtained from operated and sham-operated mice were fixed in methacarn and processed as described above. Additional sections were obtained in silanized glass slides and were deparaffinized with xylene. Tissues were then hydrated, endogenous peroxidase was blocked with 5% H2O2 for 30 minutes and, finally, slides were incubated with a polyclonal anti-Cx43 antibody 1:100 (Zymed) overnight at 4ºC. This was followed by incubation with secondary anti-rabbit antibody (Zymed, 1:1000) for 2 hours at room temperature. In addition, signal amplification was performed with TSA kit (Perkin-Elmer, Life Sciences, Boston, MA, USA). Nuclei were counter-stained with propidium iodide (1:1000) and slides were mounted with Pro-long Antifade kit (Molecular Probes). Image captures were performed in a confocal microscope Axiovert 100M LSM 510.

Western blot analysis. Samples weighing 40 mg each were homogenized directly in a buffer (1M Tris-HCl buffer, pH 6,8; 10% SDS; 10% glycerol). Later, Tris-HCl buffer (1M, pH 6,8, containing 10% SDS, 10% glycerol, 1M DTT, and 10 mM PMSF) was added and the suspension was centrifuged (13000 rpm, 15 minutes, 4°C). The supernatant was stored at –20ºC until analysis. Total protein concentration was quantified with Bio-Rad Protein Assay reagent before performing the electrophoresis. Then 150□g were loaded and submitted to electrophoresis in a

12% SDS-polyacrylamide gel (48mA, 50 min). After the electrophoresis, proteins were transferred onto polyvinvlidene difluoride membrane (PVDF) (Bio-Rad Labs, Hercules, California) in semi-dry conditions. To block unspecific binding sites, the membrane was incubated with 5% skimmed milk, for 2 hours at room temperature. The membrane was then incubated overnight with rabbit polyclonal anti-Cx43 antibody (1:500, in 2% skimmed milk, Zymed Laboratories, San Francisco, CA, USA) at 4ºC. Following the primary antibody incubation, a horseradish peroxidase anti-rabbit IgG (1:1000, in 2% skim milk) was incubated for 2 hours at room temperature. At last, Cx43 was revealed with a solution containing DAB-nickel (Sigma) and hydrogen peroxide. Equal protein amounts were visualized by Commassie Blue gel staining.

Statistical analysis. Statistical evaluation of morphometric data was performed with one-way ANOVA-test. Values of $P<0.05$ were considered significant.

Results

Nephrectomy increased the relative weight of kidney and cell proliferation.

All animals that have their kidneys excised presented an increase in kidney relative weight, compared with sham operated mice (Fig. 1, $p<0.0001$). These differences were noted at all times studied after surgery. This effect is probably due to an early increase in the kidney's cell proliferation after nephrectomy (Fig. 2), mostly at 24 ($p=0.0435$) and 48 hours ($p=0.0459$). Positive BrdU cells were mostly tubular epithelium of renal cortex region, whereas in the medullary region, a weak staining was seen and rarely glomeruli and Bowman's-capsule cells were stained. On the other hand, no differences were observed after 7 or 30 days of intervention and SHAM mice presented rare cells stained with BrdU as expected.

Morphometrical alterations after nephrectomy.

Morphometric study was performed using stereological method which permitted determination of fraction and volume occupied by different histological structures from renal parenchyma. These results are presented in table 1 and no differences were observed in any structure examined. Notwithstanding, the volume and area occupied by glomeruli were higher in nephrectomized animals evaluated after 30 days (figures 3 and 4), indicating an increase renal function.

Cx43 staining pattern was first evidenced in sham mice, where not only a punctuated pattern in membrane of proximal tubular cells was noted, but also cytoplasmic and nuclear Cx43 localization was observed (Fig. 5A). Regarding Malpighi corpuscles in sham-operated mice, Cx43 was expressed mostly at membrane-membrane cell contact as seen in Fig. 5A.

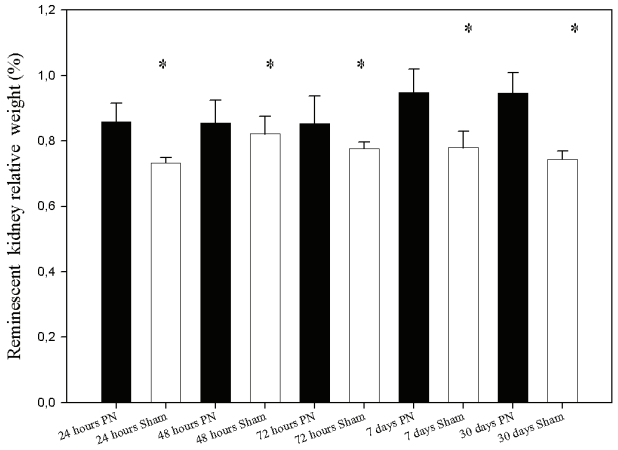

Fig. 1 – Relative weight of remnant kidney after nephrectomy. Nephrectomized animals presented increased Renal relative weight at all times after nephrectomy compared with sham operated animals. PN=post-nephrectomy. *P<0.0001.Connexin 43 immunohistochemical localization.

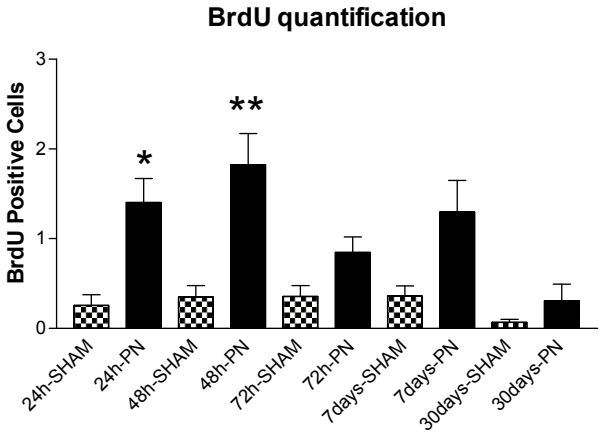

Fig. 2 – Positive BrdU cells in remnant kidney after nephrectomy. Nephrectomized mice presented increased cell proliferation only at 24 ($p=0.0435$) and 48 hours ($p=0.0459$) after surgery. PN = post-nephrectomy. *P<0.05.

Twenty-four hours after nephrectomy, Cx43 were localized mostly at membranes (Fig. 5B) in proximal tubular cells, whereas those intense nuclear and cytoplasmic staining pattern characterized in the sham-operated mice were absent. Forty-eight hours after nephrectomy, only few punctuated plaques were noted, which correlates with increased cell proliferation at this time point (Fig. 5C and 5D). Interestingly, at 72 hours, Cx43 was re-expressed and again showed a cytoplasmic and membrane pattern (Fig. 5E). As expected, 7 and 30 days after nephrectomy, Cx43 levels were similar to sham-operated animals, with cytoplasmic and punctuated membrane pattern (Fig. 7). Regarding Malpighi corpuscles, a surprisingly nuclear Cx43 pattern were

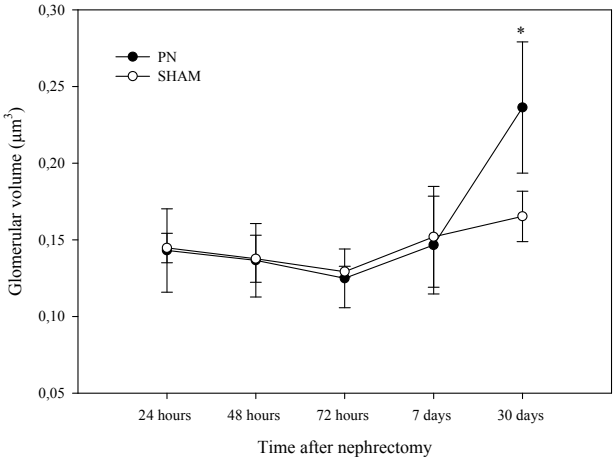

Fig. 3 – Glomeruli volume quantification in nephrectomized and SHAM-operated mice. An increase in glomerulus volume was noted at 30 days after nephrectomy (*P<0.0001). PN = post-nephrectomy.

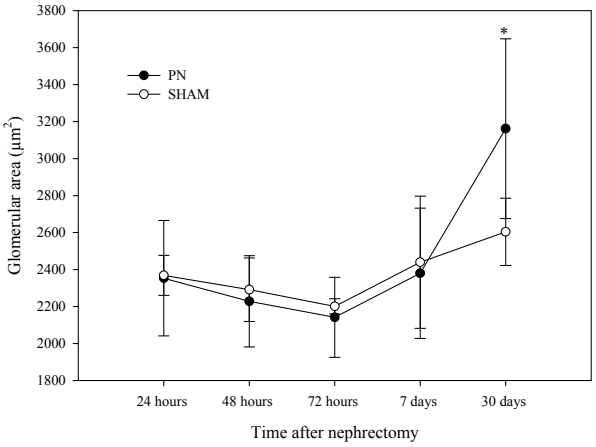

Fig. 4 – Glomeruli area quantification in nephrectomized and SHAM-operated mice. An increase in glomerulus area was noted at 30 days after nephrectomy (*P<0.0002). PN = post-nephrectomy.

observed after 24 hours of nephrectomy (Fig. 6B). In addition, after 48 hours, Cx43 was less detected and were at cytoplasm mostly (Fig. 6C). While proximal tubular cells at 72 hours presented again a Cx43 membranous pattern, Malpighi corpuscles showed a punctuated cytoplasmic staining (Fig. 6D).

Nephrectomy alter Connexin 43 phosphorylated isoforms.

Connexin43 could be phosphorylated at several different sites in its carboxy-terminal (11) and western blot technique and a suitable antibody were very useful to detect these types of changes regarding Cx43. First of all, the sham-operated mice in this study presented two easily recognizable bands, known as P0 (unphosphorylated) and P2 (hyperphosphorylated). However, no P1 (phosphorylated) were noted (Fig. 8). On the other hand,

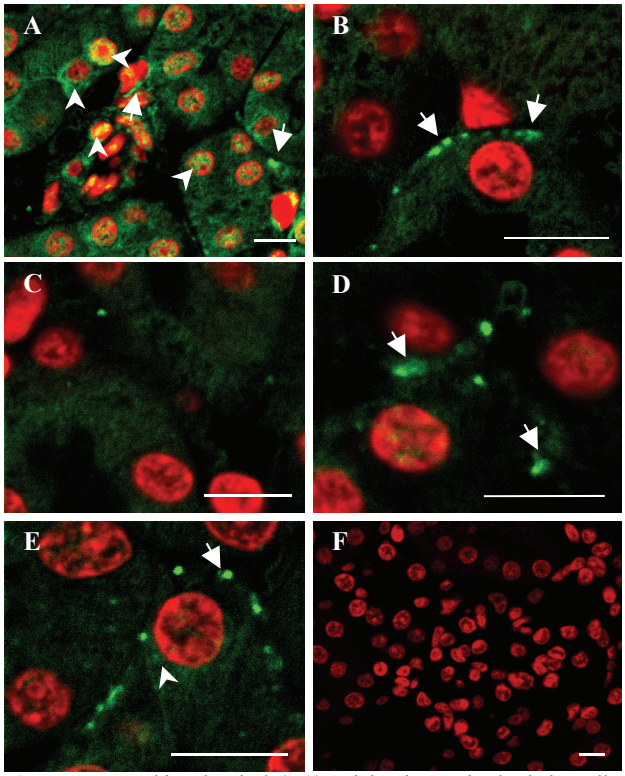

Fig. 5 – Immunohistochemical Cx43 staining in proximal tubular cells. (**A**) SHAM mice sacrificed at 24 hours. It is possible to observe connexins in all compartments (nuclear region, cytoplasm and membrane). (**B**) Mice sacrificed 24 hours after nephrectomy. Cx43 was regarded only at membrane region. (**C**) Mice sacrificed 48 hours after nephrectomy. Absence of staining. (**D**) Mice sacrificed 48 hours after nephrectomy. Cytoplasmatic staining is observed. (**E**) Mice sacrificed 72 hours after nephrectomy. Cytoplasmatic and punctual at membranes were observed. (**F**) Negative control. Arrowhead shows cytoplasmatic or nuclear staining. Arrow point to membrane staining. Nucleus was stained with Propidium iodide. Bar = 10µm.

heart tissue has been used as internal positive control of Cx43 expression, since it was the organ in which Cx43

As expected, nephrectomized mice lost Cx43 P0 and P1 bands at 24 and 48 hours, slowly increasing Cx43 expression after 72 hours (Fig. 8). In addition, P2 bands were increased at all times evaluated after nephrectomy. These data were in accordance with Cx43 imunohistochemistry, since low levels of Cx43 were detected in proximal tubular cells and Malpighi corpuscles at 24 and 48 hours. In addition, almost no degraded bands were observed from 24 until 72 hours after nephrectomy. Regarding seven and thirty days after nephrectomy, Cx43 apparently showed similar pattern to sham-operated mice, owing to higher intensity of P0 and P1 state and a lower P2 (hyperphosphorylated) bands. In brief, Cx43 western blot correlated well with the immunohistochemical data, showing time-dependently alterations of Cx43 expression in compensatory renal growth.

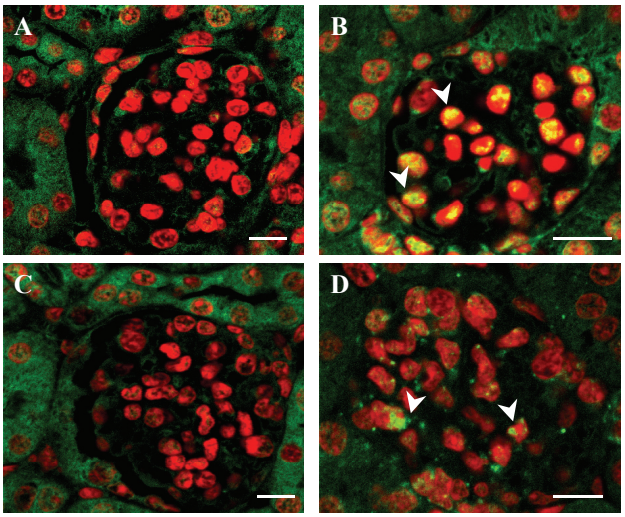

Fig. 6 – Immunohistochemical Cx43 staining in Malpighi corpuscles. (**A**) SHAM mice sacrificed with 24 hours. Cx43 was detected in high levels. (**B**) Mice sacrificed 24 hours after nephrectomy. Cx43 was regarded intensely stained colocalized with nuclear region. (**C**) Mice sacrificed 48 hours after nephrectomy. Cx43 was observed in cytoplasmatic region. (**D**) Mice sacrificed 72 hours after nephrectomy. Cytoplasmatic staining is observed. Arrowhead shows cytoplasmatic or nuclear staining. Arrow point shows membrane staining. Nucleus was stained with Propidium iodide. Bar = 10µm.

was most expressed in mammals. In this study, the heart showed the greatest Cx43 expression, and it was also possible to note three characteristic bands of degradation products (20 to 30 kDa).

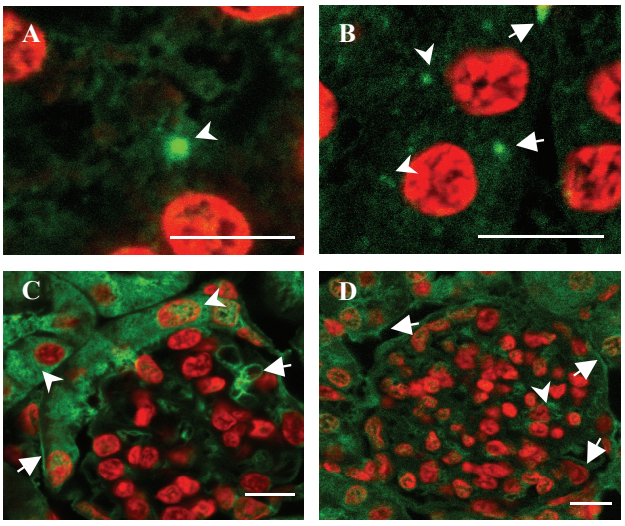

Fig. 7 – Immunohistochemical Cx43 staining in proximal tubular cells (PTC) and glomerulus. (**A**) Mice sacrificed 7 days after nephrectomy. Cx43 was regarded intensely stained in a cell-cell contact area in PTC. (**B**) Mice sacrificed 30 days after nephrectomy. Cx43 was observed in cytoplasmatic region and in cell-cell contact area of PTC. (**C**) Mice sacrificed 7 days after nephrectomy. Cytoplasmatic and membrane staining is observed in glomerulus. (**D**) Mice sacrificed 30 days after nephrectomy. Arrowheads show cytoplasmatic or nuclear staining. Arrow point to membrane staining. Nucleus was stained with Propidium iodide. Bar = 10µm.

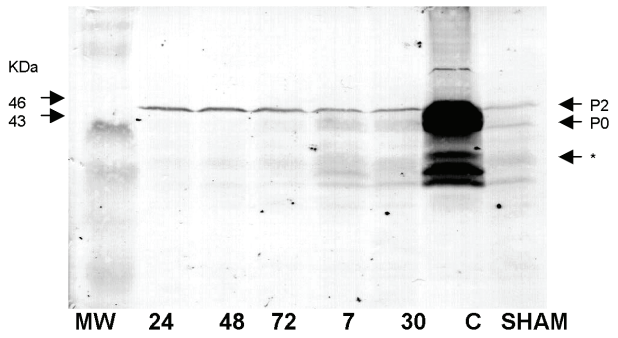

Fig. 8 – Western blot of Cx43. The immunoblot was resolved using 150 µg of protein per lane. **MW:** molecular weight marker; **C**: control heart from mice; **24, 48 and 72 hours; 7 and 30 days** after nephrectomy; **SHAM:** non-nephrectomyzed mice. **P2**: hyper-phosphorylated isoform; **P0:** non-phosphorylated isoform. "*" represents degradation products. Immunoblots were made in duplicated with the same results.

Discussion

The mechanism of compensatory renal growth of the remnant kidney after surgical excision is not fully understood. As the earliest change, glomerular hypertrophy leads to increased glomerular filtration. Depending on how much renal tissue was lost, hyperplasia could happen. Agreeing with this information, Megyesi and coworkers (14) suggest that hyperplasia is induced by the renal injury, whereas the hypertrophy is associated with the increased demand for work capacity. Here we characterized the left kidney proliferative response and Cx43 expression after surgical excision of the right kidney.

In this study, a significant increase in the remnant kidney weight was observed during compensatory renal growth. As soon as 24 hours, the relative weight increased approximately 11% in comparison to the sham group. Furthermore, this parameter was regarded in a steady state during the first 72 hours after nephrectomy, culminating with a peak of 21% growth 30 days after nephrectomy. In addition, both glomeruli area and volume of nephrectomized mice were increased possibly indicating either hypertrophy or hyperplasia. According to early reports, the increase in mass observed a posteriori is an adaptation to higher tax of filtration per renal corpuscle remnant, which could be increased as much as 60% (28). This data corroborated with Megyesi and coworkers (14), whom characterized this early increased renal weight as being principally due to hypertrophy.

The cellular proliferation during the process of compensatory renal hyperplasia was demonstrated here by 5-bromodeoxyuridine (BrdU) immunohistochemical detection; a known marker of the synthesis phase of cell cycle. In this work, increased cell proliferation was easier to observe at the kidney's cortical region, mostly being epithelial cells from proximal tubules. Moreover, nephrectomized mice showed increased BrdU positive nuclei as soon as 24 and 48 hours post-nephrectomy, which characterized the presence of hyperplasia.

According with our data, Li and coworkers (12) showed a significant increase of PCNA positive cells after two days from total unilateral urethral obstruction.

Recently, a transient decrease of gap junctional intercellular communication was demonstrated during compensatory renal growth in mice (12). The authors claimed that these alterations were due to altered connexin43 phosphorylation, but they didn't correlate those data with cell proliferation. Generally, Cx43 isolated from adjacent quiescent mammalian cells and resolved by SDS-PAGE migrates as a faster, nonphosphorylated (NP) form and one or more heavy phosphorylated isoforms. Normally two predominant phosphoisoforms (P1 and P2) are observed, mostly being on serine residues (11). Connexin43 was down-regulated during certain phases of cell cycle (22), mostly through specific phosphorylations in the carboxy-terminal region of Cx43, leading to increased Cx43 internalization and degradation by lysossomes (11). Another interesting data came from early works (10,27) where the change in gap junctional intercellular communication correlated with a redistribution of Cx43 from the plasma membrane to cytoplasmic structures.

Although phosphorylation does not appear to be absolutely required for eventual formation of Cx43 gap junction plaques, observations that Cx43 is phosphorylated prior to its arrival at plasma membrane (17), suggest that phosphorylation may modulate Cx43 trafficking and/or the assembly or disassembly of gap junctions. Accordingly to Li and coworkers (12), Cx43-P2 form is decreased and was characterized as a functional form in kidney after unilateral urethral obstruction, once a diminished gap junction communication was observed by freeze fracture technique. Our results clearly show that Cx43-P2 is the predominant connexin isoform during the first 72 hours after nephrectomy. Corroborating with this data, reduced degradation products were observed from 24 until 72 hours evidencing a decreased Cx43 turnover.

The available data indicate that Cx43 is synthesized at the rough endoplasmic reticulum (RER), transported then to Golgi apparatus, and ultimately trafficked to plasma membrane, where it forms gap junction plaques. The assembly of connexins into hexamers has been reported to occur in the trans-Golgi network (4,18). Our data suggest that during renal cells proliferation peak observed at 48 hours, Cx43 was retained in the cytoplasm or co-localized into the nucleus. In addition, the phosphorylated Cx43 pattern seen here was altered during 72 hours after nephrectomy. In addition, we noted the striking appearance of a unique, slow-migrating Cx43 isoform, coupled with a nearly complete disappearance of the faster-migrating Cx43 phospho-isoforms, characteristically presented in non-mitotic cells (9,27). It is believed that early phosphorylation events could be involved in the proper trafficking of Cx43 form from RER, Cx43 oligomerization into connexons, or the exit of Cx43 connexons from the trans-Golgi network. A reduced Cx43-P0 and P1 isoforms observed here demonstrated that early phosphorylation events could be transient and necessary to maintain these connexins in the cytoplasm.

In conclusion, our results suggest that Cx43 phosphorylation results in the retention of Cx43 in cytoplasm and its increased degradation during compensatory renal hyperplasia/hypertrophy.

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