

行政院國家科學委員會專題研究計畫 期中進度報告

H0-1 與 bcl-2 基因載入於缺血性腎衰竭之氧化壓力與細胞 凋亡評估(1/2)

計畫類別：個別型計畫

計畫編號：NSC93-2314-B-002-154-

執行期間：93 年 08 月 01 日至 94 年 07 月 31 日

執行單位：國立臺灣大學醫學院醫學系

計畫主持人：賴明坤

共同主持人：鄭劍廷

報告類型：精簡報告

處理方式：本計畫可公開查詢

中 華 民 國 94 年 5 月 16 日

Adenovirus-Mediated bcl-2 Gene Transfer Inhibits Renal Ischemia/Reperfusion Induced Tubular Oxidative Stress and Apoptosis

Chien Chiang-Ting^a, Chang Tzu-Ching^c,
Tsai Ching-Yi^a, Shyue Song-Kuen^c
and Lai Ming-Kuen^{b,*}

^aDepartments of Medical Research
and ^bMedicine, National Taiwan University Hospital and
National Taiwan University College of Medicine, Taipei
^cInstitute of Biomedical Sciences, Academia Sinica,
Taipei, Taiwan, ROC

*Corresponding author: Lai Ming-Kuen,
mingkuen@ntuh.gov.tw

Ischemia/reperfusion induces oxidative injury to proximal and distal renal tubular cells. We hypothesize that Bcl-2 protein augmentation with adenovirus vector mediated bcl-2 (Adv-bcl-2) gene transfer may improve ischemia/reperfusion induced renal proximal and distal tubular apoptosis through the mitochondrial control of Bax and cytochrome C translocation. Twenty-four hours of Adv-bcl-2 transfection to proximal and distal tubular cells *in vitro* upregulated Bcl-2/Bax ratio and inhibited hypoxia/reoxygenation induced cytochrome C translocation, O_2^- production and tubular apoptosis. Intra-renal arterial Adv-bcl-2 administration with renal venous clamping augmented Bcl-2 protein of rat kidney *in vivo* in a time-dependent manner. The maximal Bcl-2 protein expression appeared at 7 days after Adv-bcl-2 administration and the primary location of Bcl-2 augmentation was in proximal and distal tubules, but not in glomeruli. With a real-time monitoring O_2^- production and apoptosis analysis of rat kidneys, ischemia/reperfusion increased renal O_2^- level, potentiated proapoptotic mechanisms, including decrease in Bcl-2/Bax ratio, increases in caspase 3 expression and poly-(ADP-ribose)-polymerase fragments and subsequent proximal and distal tubular apoptosis. However, Adv-bcl-2 administration significantly enhanced Bcl-2/Bax ratio, decreased ischemia/reperfusion induced O_2^- amount, inhibited proximal and distal tubular apoptosis and improved renal function. Our results suggest that Adv-bcl-2 gene transfer significantly reduces ischemia/reperfusion induced oxidative injury in the kidney.

Key words: Apoptosis, bcl-2, gene transfer, ischemia/reperfusion, kidney, oxidative stress

Received 24 June 2004, revised 4 December 2004 and accepted for publication 21 December 2004

Introduction

Complete or partial cessation (ischemia) followed by restoration of blood flow (reperfusion) impairs many organs, such as the heart, brain, liver and kidney (1–4). Ischemia/reperfusion injury induces burst release of reactive oxygen species (ROS) (1–4), which contribute to abnormal signal transduction or cellular dysfunction (5,6) and initiate the cascade of apoptosis/necrosis (2,7).

Mitochondrial dysfunction following oxidative injury is an early event in apoptotic cell death, since the apoptogenic factor, cytochrome C, is released into the cytoplasm (7). Once this translocation occurs, cytochrome C binds to another cytoplasmic factor, Apaf-1, and the formed complex activates the initiator caspase-9 that in turn activates the effector caspases, of which caspase-3 is a prominent member (8). Release of cytochrome C from the mitochondria can be triggered by the proapoptogenic Bax (9). While Bax has been shown to trigger cell death (10), the anti-apoptotic Bcl-2 can block cytochrome C release and caspase activation (11). In the kidney, ROS are produced in significant amounts in renal proximal rather than distal tubular epithelium under ischemia/reperfusion or hypoxia/reoxygenation conditions (2). The increased ROS production enhances Bax/Bcl-2 ratio (2,12), caspase 3 (CPP32) expression (2,13) and poly-(ADP-ribose)-polymerase (PARP) fragments (14), and subsequently resulted in severe apoptosis, including increases in DNA fragmentation and apoptotic cell number in renal tubules.

Anti-oxidant Bcl-2 (15) resides in the mitochondria and prevents activation of the effector caspases by mechanisms such as blockade of the mitochondria permeability transition pore (MTP) opening (16), or by functioning as a docking protein (17). Over-expression of Bcl-2 can block both apoptosis and necrosis (18,19), and protect ischemic tissue against reperfusion induced oxidative stress (20). Therefore, the application of local bcl-2 gene transfer into the kidney to augment Bcl-2 protein seems promising as a therapeutic strategy for reduction of renal ischemia/reperfusion injury.

In this study, a replication-defective adenovirus vector-mediated gene transfer was used to augment Bcl-2 in rat kidney to obviate concerns regarding the purity of the

enzyme and preparation or fluctuations in Bcl-2 protein delivery. Our results demonstrate that proximal and distal tubules enriched in Bcl-2 are more resistant to the damaging effects of ischemia/reperfusion by downregulation in ROS amount, Bax/Bcl-2 ratio, cytochrome C release and subsequent reduction of apoptotic cell death.

Methods

Preparation of recombinant adenoviral vector

A human bcl-2 cDNA containing the entire coding sequence was subcloned into the E1 and E3 deleted adenovirus shuttle plasmid, which contains a promoter of the human phosphoglycerate kinase (Adv-pgk). A recombinant adenovirus (Adv-bcl-2) was generated by homologous recombination and amplified in human embryonic kidney (HEK) 293 cells as previously described (21). The human bcl-2 cDNA was kindly provided by Dr. J. Y. Yen of the Institution of Biomedical Science, Academia Sinica (22). Virus stocks were purified by CsCl density gradient centrifugation, aliquoted and stored at -80°C . Viral titers, plaque-forming units (pfu), were determined by plaque-forming assay that HEK 293 cells were infected with serially diluted viral preparations and then overlaid with low melting-point agarose after infection. Numbers of plaques formed were counted within 2 weeks.

Animals

Female Wistar rats (200–250 g) were housed at the Experimental Animal Center, National Taiwan University, at a constant temperature and with a consistent light cycle (light from 7 AM to 6 PM). Food and water were provided ad libitum. All surgical and experimental procedures were approved by the animal care and experimental protocols were in accordance with the guidelines of the National Science Council of the Republic of China (NSC 1997).

Gene transfer to renal proximal (PT) and distal tubules (DT)

Under sodium pentobarbital (40 mg/kg, i.p.) anesthesia, kidneys were flushed with 20 mL of ice-cold Krebs-Henseleit-saline buffer via an aortal catheter. Specific isolation of PT and DT was performed as previously described (2). Each of the two types of cells was identified and cultured (2,23). For infection, 3×10^5 PT and DT cells were seeded on a 10-cm² Petri dish and treated with Adv-pgk or Adv-bcl-2 at 10^7 pfu for 24 h. Immunohistochemistry for expression of Bcl-2 protein in PT and DT cells were used to evaluate the transfection efficiency. After 24 h incubation, cells were collected and plated on slides. The slides were washed, fixed and blocked for 20 min in Tris-buffered saline containing 10 mL/L goat serum, then incubated with monoclonal mouse anti-human Bcl-2 (Transduction) diluted 1:100 in PBS overnight. The slides were stained by an avidin-biotinylated horseradish-peroxidase procedure using a commercially available kit (ABC Elite; Vector Laboratories, Burlingame, CA, USA).

Induction of hypoxia/reoxygenation of the renal tubule cells was performed as described (2). The cultures were first placed in an atmosphere of 95% O₂/5% CO₂ at 37°C for 30 min. Hypoxia was achieved by gassing with 95% N₂/5% CO₂ for 15 min, whereas reoxygenation was performed by reintroduction of 95% O₂/5% CO₂ for 30 min. For determination of the number of apoptotic cells in culture, the cells were fixed with 70% ethanol, stained with propidium and counted with FACSCalibur (Becton Dickinson, San Jose, CA). Cell viability was counted with a Trypan blue dye exclusion test. The amounts of ROS in PT and DT cells (10^6 cells/mL) were detected by the lucigenin-enhanced ROS test (2).

Intra-renal arterial gene delivery

For direct gene delivery, an intra-renal arterial catheter was performed via the left femoral artery (2). Under avertin anesthesia (400 mg/kg, Acros Organics, NJ), one PE10 tubing was introduced into the left renal artery from the left femoral artery via the aorta. Based on our preliminary results, 10^8 pfu of Adv permitted efficient gene delivery. Adv-pgk or Adv-bcl-2 of 10^8 pfu in 0.2 mL of saline was infused into the left kidney at a rate of 20 $\mu\text{L}/\text{min}$ via the intra-renal arterial catheter with clamping of the renal vein for 10 min. To ascertain the transgene expression at the region of Adv infusion, we infused adenoviruses containing a green fluorescent protein (GFP) gene, Adv-GFP, into the left kidney and examined the GFP expression in rat kidneys 3–28 days later. High levels of GFP were visualized under UV in the arterial lining cells and tubular cells and maximal fluorescence was found in the left kidney sections at day 7 but not in the right kidney. These results confirmed the uptake and expression of Adv by the left kidneys. Immunohistochemistry for expression of Bcl-2 protein in the kidney *in vivo* were used to evaluate the transfection efficiency. After 7 days of infection, the kidneys were removed and fixed in 10% neutral buffer formalin. Tissue sections were stained with monoclonal mouse anti-human Bcl-2 (transduction) diluted 1:400 and followed by an avidin-biotinylated horseradish-peroxidase procedure using a commercially available kit (ABC Elite; Vector Laboratories). The transfection efficiency was evaluated by measuring Bcl-2 positive area (PT, DT and glomeruli) on the kidney section.

After Adv-pgk or Adv-bcl-2 injection into the left kidney, the incision was closed in layers with 3.0 suture (Ethicon), and the animals were allowed to recover. Rats were sacrificed by overdose of sodium pentobarbital at indicated time after Adv-pgk or Adv-bcl-2 administration. In some rats, renal glomeruli were isolated by graded sieving (250, 150 and 75 μm) (24) and PT and DT cells were isolated as described above. The isolated proteins from separated glomeruli, PT and DT were used to determine Bcl-2 protein expression after Adv-bcl-2.

Induction of renal ischemia

The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and were tracheotomized. For induction of ischemia in the left kidney, the right kidney was removed and the left renal artery was clamped 45 min with a small vascular clamp. Sham-operated animals underwent similar operative procedures without occlusion of the left renal artery. Reperfusion was initiated by removal of the clamp for 1–24 h. After ischemia/reperfusion insults, arterial blood were collected for renal functional determination. Blood urea nitrogen (BUN) and plasma creatinine were analyzed using a commercial kit from Sigma (St Louis, MO). The kidney was resected and divided into two parts. One part was stored in 10% neutral buffered formalin for immunocytochemical and *in situ* apoptotic assay, and the other was quickly frozen in liquid nitrogen and stored at -70°C for protein isolation.

In vivo chemiluminescence recording for ROS activity

The ROS generation in response to ischemia/reperfusion injury was measured from the kidney surface by intra-renal arterial infusion of a superoxide anion probe, 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one-hydrochloride (MCLA) (0.2 mg/mL/h, TCI-Ace, Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) and by use of a Chemiluminescence Analyzing System (CLD-110, Tohoku Electronic In. Co., Sendai, Japan) (2). The real-time displayed chemiluminescence signal was recognized as ROS level from the kidney surface.

In situ demonstration of superoxide generation and apoptosis formation

A nitroblue tetrazolium (NBT) perfusion method was used for localizing *de novo* ROS generation in the insulted kidney (2). Rats ($n = 3$ in

each group) were sacrificed at the end of ischemia/reperfusion injury. An 18-gauge needle connected to an infusion pump (Infors AG, CH-4103, Bottmingen, Switzerland) was inserted into the lower abdominal aorta just below the level of the renal artery. The kidneys were perfused with 37°C Hanks' balanced salt solution (flow rate, 10 mL/min; pH 7.4), and the perfusate was allowed to drain from the inferior vena cava. Once blood had been removed, NBT (1 mg/mL) was added to the solution, and the kidney was perfused for an additional 10 min at a flow rate of 5 mL/min. All unreacted NBT was removed from the kidneys by perfusion with Hanks' solution. The NBT-perfused kidney was removed and fixed in zinc/formalin (1% ZnSO₄ in 10% formalin) for histologic examination for formazan deposits.

The method for the terminal deoxynucleotidyl transferase-mediated nick-end labeling method (TUNEL) was performed as previously described (2,12,13). Sections of the kidney were stained by the methyl green and TUNEL-avidin-biotin-complex method. Twenty high-power (×400) fields were randomly selected, and the value of apoptotic cells/apoptotic cells and methyl green stained cells) was counted. The number of apoptotic cells was expressed per 100 of the tubular cells in each section.

Immunostaining for Bax, Bcl-2, and cytochrome C

Localization of Bax and Bcl-2 was detected in 10% neutral-buffered formalin fixed, paraffin-embedded 4-μm sections. After deparaffinization and hydration, the sections were heated at 95°C in pH = 7.0 10 mmol/L citrate acid buffer. The activity of endogenous peroxidase was quenched by 3% (v/v) H₂O₂ in methanol. The sections were then incubated with the primary antibody overnight at 4°C in a humid atmosphere. A rabbit polyclonal anti-human Bax (Chemicon, Temecula, CA) and monoclonal mouse anti-human Bcl-2 (Transduction, Bluegrass-Lexington, KY) were diluted at 1:400. The sections were stained by an avidin-biotinylated horseradish-peroxidase procedure using a commercially available kit (ABC Elite; Vector Laboratories). Finally, the color was developed by the 3'-Amino-9-ethylcarbazole substrate (Vector Laboratories) and counter stained with hematoxylin. Control sections were incubated with the same species normal IgG or serum at the same protein concentration as the primary antibody.

For cytochrome C staining, Adv-pgk or Adv-bcl-2 treated PT with hypoxia/reoxygenation and 5 μm of kidney cryosections were performed. These samples were incubated overnight at 4°C with the primary antibodies, cytochrome C rabbit polyclonal antibody and heat shock protein 60 (HSP60) goat polyclonal antibody (Santa Cruz Biotechnology, Inc.) and then incubated in anti-rabbit fluorescein isothiocyanate (FITC) and anti-goat rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). These immunofluorescence images were taken by a fluorescent microscopy (Leica CM1900) for tissue section and a laser scanning confocal system (MRC 1000, Bio-Rad Laboratories) for PT.

Bax, Bcl-2, cytochrome C, CPP32 and PARP expression

Cytosolic Bax translocation to mitochondria and mitochondrial leakage of cytochrome C to cytosol are required for triggering apoptotic pathway (25,26). PT and DT cells were subjected to differential centrifugation to obtain the mitochondrial and cytosolic fractions. Protein concentration was determined by a BioRad Protein Assay (BioRad Laboratories, Hercules, CA). Ten μg of protein was electrophoresed as described below. The primary antibody of polyclonal rabbit anti-human cytochrome C (Santa Cruz Biotechnology, Inc.) and polyclonal rabbit anti-human Bax (Chemicon) was used at 1:1000.

The expression of Bax, Bcl-2, caspase 3 and PARP of kidney tissue was evaluated by western immunoblotting and densitometry (2). Briefly, the total proteins were homogenized with a pre-chilled mortar and pestle in extraction buffer, which consisted of 10 mM Tris-HCl (pH 7.6), 140 mM NaCl, 1 mM phenylmethyl sulfonyl fluoride, 1% Nonidet P-40, 0.5% deoxycholate, 2% β-mercaptoethanol, 10 μg/mL pepstatin A and 10 μg/mL aprotinin. The

mixtures were homogenized completely by vortexing and kept at 4°C for 30 min. The homogenate was centrifuged at 12000 × g for 12 min at 4°C, the supernatant was collected and the protein concentrations were determined by BioRad Protein Assay (BioRad Laboratories).

The polyclonal rabbit anti-human Bax (Chemicon), monoclonal mouse anti-human Bcl-2 (Transduction), polyclonal rabbit anti-human caspase 3 (CPP32/Yama/Apopain, Upstate Biotechnology, Lake Placid, NY), monoclonal mouse anti-human PARP (Promega, Madison, WI) and monoclonal mouse anti-mouse β-actin (Sigma, Saint Louis, MI) were used. All these antibodies cross-react with the respective rat antigens.

Statistical analysis

All values were expressed as mean ± standard error mean (SEM). Differences within groups were evaluated by paired t-test. One-way analysis of variance was used for establishing differences among groups. Intergroup comparisons were made by Duncan's multiple-range test. Differences were regarded as significant if $p < 0.05$ was attained.

Results

Adv-bcl-2 transfection augmented Bcl-2 on PT and DT

To examine the role of the induction of Bcl-2 protein by Adv-bcl-2, Adv-bcl-2 was transfected into the PT and DT cultured cells for 24 h. Twenty-four hours after transfection with Adv-pgk or Adv-bcl-2, PT and DT cells were probed with monoclonal mouse anti-human Bcl-2 antibody. Immunohistochemistry with an anti-Bcl-2 antibody clearly demonstrated that Bcl-2 protein was highly expressed in the Adv-bcl-2 transfected PT (45 ± 3%) and DT (39 ± 3%) cells, but less expressed in the Adv-pgk treated PT (4 ± 1%) and DT cells (3 ± 0.5%). This indicated that human bcl-2 gene mediated by recombinant adenovirus was highly expressed in PT and DT cells.

In the basal level with Adv-pgk, the Bcl-2 protein expression of DT was 4.7 ± 0.8 times higher ($p < 0.05$) than that of PT. Adv-bcl-2 administration augmented 1.7 ± 0.3 folds of DT and 6.5 ± 1.4 folds of PT Bcl-2 expression possibly by a different efficiency. Hypoxia/reoxygenation enhanced Bcl-2 expression, and activated Bax translocation to mitochondria and cytochrome C translocation to cytosol in both PT and DT cells (Figure 1A). Over-expression of Bcl-2 protein by Adv-bcl-2 inhibited cytochrome C translocation, but not Bax translocation in both DT and PT cultures. We confirmed that increased Bcl-2/mitochondrial Bax ratio by Adv-bcl-2 transfection was efficiently inhibited hypoxia/reoxygenation induced cytochrome C release to cytosol by immunoblotting (Figure 1A) and confocal immunofluorescence (Figure 1B).

Bcl-2 augmentation inhibits DT and PT apoptosis and ROS amount

In correlation with the level of Bcl-2 protein expressed, PT and DT apoptosis and ROS release induced by hypoxia/reoxygenation was inhibited by Adv-bcl-2 administration (Figure 2). The augmented Bcl-2 present in DT and PT

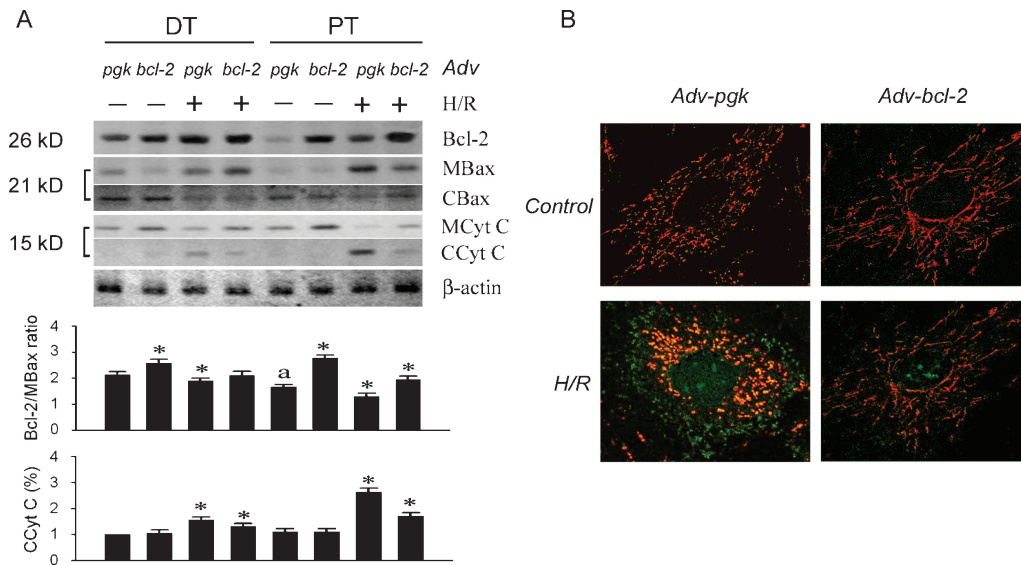


Figure 1: Augmented Bcl-2 by Adv-bcl-2 blocks cytochrome C translocation without inhibiting Bax translocation in the PT and DT cultures (n = 3 each). (A) 10 µg of total protein (Bcl-2 and β-actin), cytosolic Bax (CBax) and cytochrome C (CCyt C), mitochondrial Bax (MBax) and cytochrome C (MCyt C) was analyzed by SDS-PAGE followed by immunoblotting. In baseline condition (Adv-pgk control), Bcl-2 expression in DT was higher than in PT. Adv-bcl-2 augmented both DT and PT Bcl-2 level when compared to Adv-pgk. Hypoxia/reoxygenation (H/R) enhances Bcl-2, but also activates Bax translocation to mitochondria and Cyt C translocation to cytosol. Over-expression of Bcl-2 protein by Adv-bcl-2 gene transfer can inhibit Cyt C translocation, but not Bax translocation in both DT and PT cultures. By densitometry analysis, H/R injury decreases the Bcl-2/MBax ratio and increases the CCyt C in both DT and PT cells. Adv-bcl-2 transfection, however, increases the Bcl-2/MBax ratio in baseline level and preserves the Bcl-2/MBax ratio after H/R injury and inhibits CCyt C translocation. (B) With confocal fluorescence microscope, H/R induced a morphological change in mitochondria (red fluorescence of HSP60 marker) and increased CCyt C (green fluorescence) leakage from the impaired mitochondria in the Adv-pgk treated PT. Adv-bcl-2 treatment inhibited green fluorescence of CCyt C leakage from mitochondria. *p < 0.05 when compared to control level of each culture cells. p < 0.05 PT versus DT cells. All values were expressed as mean ± standard error mean (SEM) of three experiments.

cells was sufficient to antagonize the apoptotic and oxidant action of hypoxia/reoxygenation.

Intra-renal arterial Adv-bcl-2 increased Bcl-2 expression in the kidney in vivo

To evaluate the efficiency of adenovirus vector administration via the intra-renal arterial route, we infused Adv-GFP into the left kidney of normal rats. After 0–28 days of administration, the green fluorescent intensity was found in the renal vessels and tubules (Figure 3B, C) of Adv-GFP kidney, but not found in the Adv-pgk treated kidney (Figure 3A).

Next, we infused Adv-bcl-2 into the left kidney and determined Bcl-2 protein level 0–28 days after administration. With Bcl-2 immunocytochemical analysis, the PT and DT cells (Figure 3E), especially in PT cells (Figure 3F), are highly stained by Bcl-2. The transfection efficiency of Adv-bcl-2 in the PT, DT and glomerular area of the kidney *in vivo* was $23.5 \pm 6.7\%$, $7.6 \pm 2.4\%$ and $0.3 \pm 0.1\%$, respectively. With western blotting analysis, compared with Adv-pgk or saline control, Adv-bcl-2 augmented Bcl-2 in a time-dependent manner (Figure 3G). Maximal augmentation of 4–5 fold of renal Bcl-2 was noted post 7 days transfection. The augmented Bcl-2 expression was primarily located in the DT (4.5 ± 0.7 folds) and PT (12.0 ± 2.1 folds) tubules, not glomeruli by western blots (Figure 3H).

Adv-bcl-2 reduced ischemia- and reperfusion-induced kidney ROS levels

Previous studies indicate bcl-2 transfer confers anti-oxidant and anti-apoptotic potential against oxidative stress (20). We therefore evaluated the effects of Adv-bcl-2 administration on $O_2^{\cdot-}$ levels and apoptosis cell death. Continuous infusion of MCLA into a control kidney displayed a basal $O_2^{\cdot-}$ level at 500–800 counts/10 s. In ischemic kidneys with Adv-pgk, an increase in $O_2^{\cdot-}$ value (1740 ± 280 counts/10 s) was observed and was maintained at the increased level for 45 min (Figure 4, trace 1). In reperfused kidneys with Adv-pgk, the $O_2^{\cdot-}$ level was further enhanced to 4120 ± 570 counts/10 s and maintained at the high level for 4–6 h. In the kidneys treated with Adv-bcl-2 (Figure 4, trace 2), the level of $O_2^{\cdot-}$ in the control, ischemia and reperfusion stages were 550 ± 90 , 1120 ± 190 and 1490 ± 245 counts/10 s, respectively. Adv-bcl-2 augmented Bcl-2 protein significantly inhibited 55% and 75% of $O_2^{\cdot-}$ production in the ischemia and reperfusion stages.

In situ localization of $O_2^{\cdot-}$ formation and proapoptotic Bax expression

We used an NBT staining method for localization of $O_2^{\cdot-}$ production in renal tissue (2). NBT is reduced to an insoluble formazan derivative upon exposure to $O_2^{\cdot-}$ and the

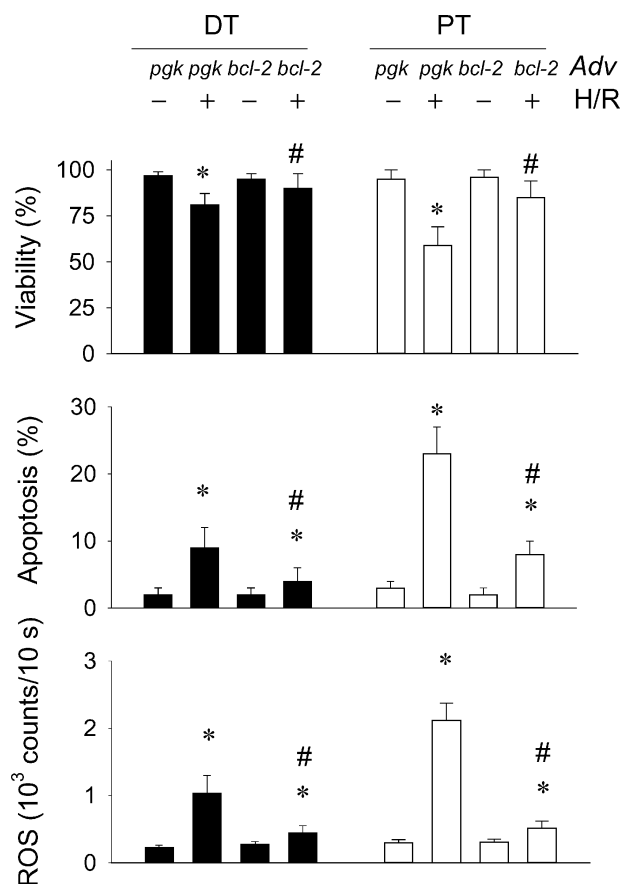


Figure 2: Effect of hypoxia/reoxygenation (H/R) on PT and DT cells. Isolated PT and DT cells (n = 3 each) were subjected to 15 min of hypoxia (95% N₂/5% CO₂) followed by 30 min of reoxygenation (95% O₂/5% CO₂). Cell viability and tubular apoptosis were measured by Trypan blue exclusion and flow cytometry. Results are mean ± SEM of three experiments. H/R to PT and DT cells produced a statistically significant (p < 0.05) decrease in cell viability and an increase in tubular apoptosis and ROS generation. Adv-bcl-2 administration to PT and DT cells greatly protected cells from H/R injury in both PT and DT cells. *p < 0.05 versus Adv-pgk treated cells without H/R injury. #p < 0.05 versus cells with H/R insult.

blue-colored formazan is detectable in tissue by light microscopy. O₂⁻ generation was not seen in kidneys without injury. There was significant O₂⁻ production (blue deposits) in ischemic kidneys after 4 h of reperfusion (Figure 5B, D). The NBT deposits were located mainly in the PT and sparsely in DT. In rats treated with Adv-bcl-2, significant decreases in NBT deposits in PT cells were observed (Figure 5C, E). With double staining of proapoptotic Bax expression in the NBT treated kidneys, Bax was not present in the kidneys without injury (data not shown), but was highly stained in the PT and DT after ischemia/reperfusion injury (Figure 5B). Both NBT and Bax stains were highly expressed in the insulted PT. Adv-bcl-2 reduced NBT deposits

and Bax expression, indicating a reduction of oxidant and apoptotic action by augmented Bcl-2 (Figure 5C).

Adv-bcl-2 gene transfer reduced ischemia/reperfusion-induced kidney apoptosis

The expression of Bax, Bcl-2, CPP32 and PARP in the kidney samples after ischemia/reperfusion was assessed by immunoblotting (Figure 5A). The expression of Bcl-2 was detected in Adv-pgk treated kidneys and was apparently enhanced after 7 days of Adv-bcl-2 administration. Bcl-2 expression was mildly increased by ischemia/reperfusion. Bcl-2 expression was significantly augmented by ischemia/reperfusion in the Adv-bcl-2 treated kidneys. Expression of Bax but not of CPP32 and PARP was detected in Adv-pgk and Adv-bcl-2 treated kidneys. The expression of three proteins was significantly increased after ischemia/reperfusion. The enhanced Bax, CPP32 and PARP expression by ischemia/reperfusion was significantly inhibited by Adv-bcl-2 treatment.

The apoptotic cells were distinguished by their brownish stained nuclei. Apoptotic cells were not detected or were only rarely present in sections from Adv-pgk or Adv-bcl-2 treated kidney. In kidneys subjected to ischemia/reperfusion, increased numbers of apoptotic cells were detected in both PT (22.0 ± 4.1%) and DT (7.3 ± 1.8%) (Figure 5D). After 7 days of Adv-bcl-2 administration, augmented Bcl-2 expression significantly (p < 0.05) reduced the number of apoptotic cells in both PT (3.6 ± 0.6) and DT (1.4 ± 0.3%) cells (Figure 5E). We have also confirmed that Adv-bcl-2, not Adv-pgk treatment inhibited green fluorescence cytochrome C leakage from mitochondria (red-fluorescence HSP60, a mitochondrial marker) (Figure 5F–I), indicating inhibition of cytochrome C translocation by Adv-bcl-2.

After 4 and 24 h of reperfusion, increased BUN and plasma creatinine levels were observed in Adv-pgk and Adv-bcl-2 rats subjected to 45-min unilateral renal ischemia with right kidney removal. However, the increased level of BUN and creatinine in Adv-bcl-2 group was significantly lower than that in Adv-pgk group (Figure 6).

Discussion

The current study indicates that intra-renal arterial infusion of Adv-bcl-2 is effective in augmenting Bcl-2 expression in kidneys and in reducing tubular apoptosis by ischemia/reperfusion injury. In DT and PT cultures *in vitro*, augmented Bcl-2 can increase Bcl-2/Bax ratio, inhibit cytochrome C translocation to cytoplasm and reduce ROS production and apoptotic cell death in hypoxia/reoxygenation injury. In rat kidney *in vivo*, the augmented Bcl-2 protein highly expressed in DT and PT cells, not glomeruli of rat kidney *in vivo* confers a selective protection against ischemia/reperfusion induced tubular oxidative stress and apoptosis.

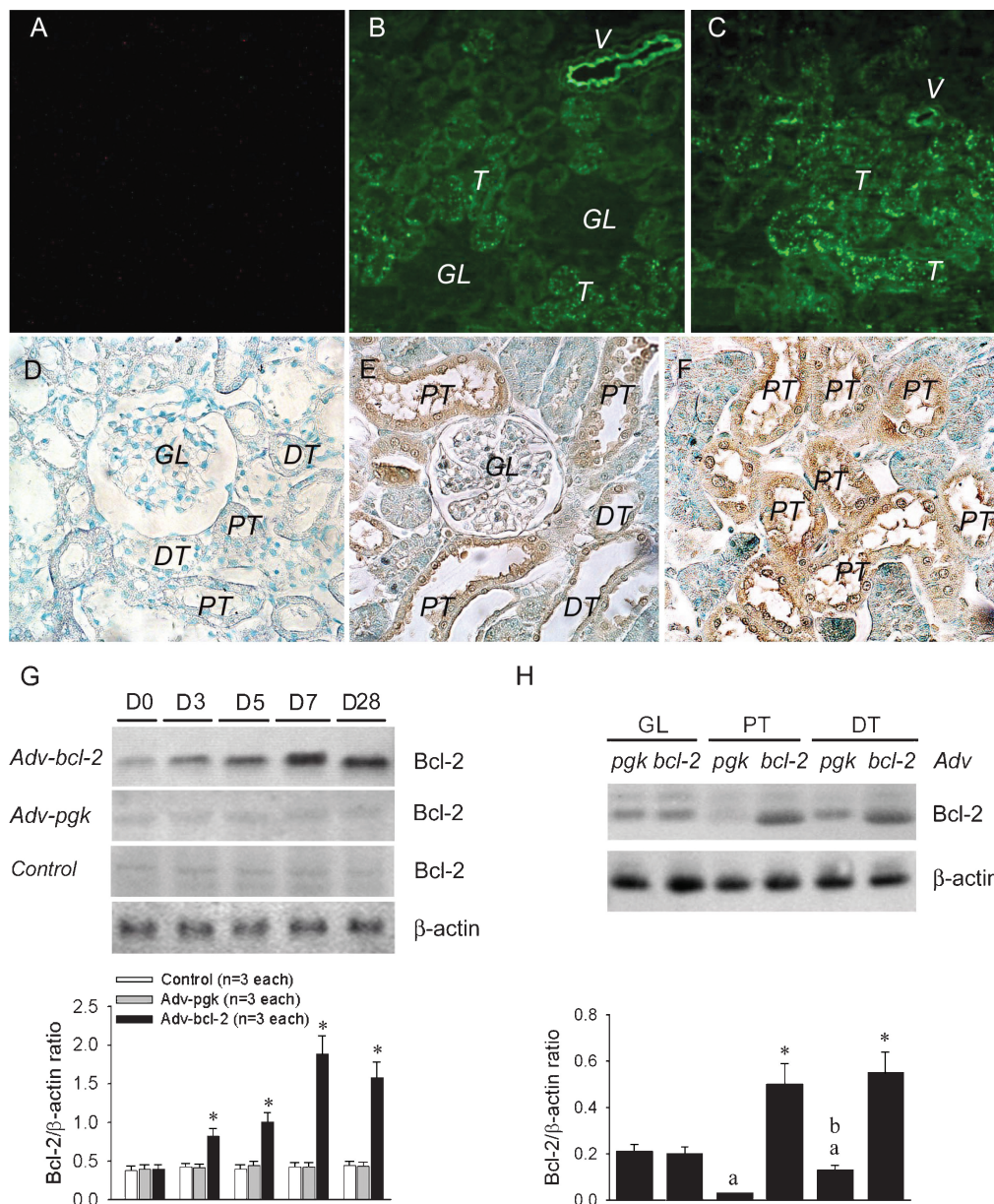


Figure 3: Effect of Adv-bcl-2 gene transfer in the kidney. After 3 (n = 4) (B) or 7 days (n = 4) (C) of intra-renal arterial administration of Adv-GFP, renal tubules (T) and some renal vessel (V) but not glomeruli (GL) display high fluorescence intensity. There is no fluorescent intensity in the control kidney with Adv-pgk treatment (A). With immunohistochemical analysis for confirmation of successful transfection and subsequent protein transcription of bcl-2 gene, the renal proximal tubules (E and F) and distal tubules (E) display a positive brownish color in the kidney section post 7 days of transfection (n = 4). The kidney (n = 4) with Adv-pgk transfer does not display any brownish-colored stain after 7 days of transfection (D). The effect of Adv-bcl-2, Adv-pgk and saline (control) treatment on renal Bcl-2 protein levels post 0–28 days (D0–D28, n = 3 each) transfection is displayed in G. Adv-bcl-2, but not Adv-pgk and control, increases the Bcl-2 protein expression in the rat kidney and a maximal expression is found at day 7. (H): Adv-bcl-2 not Adv-pgk enhances Bcl-2 protein expression primarily in the PT and DT, but not in GL post 7 days of transfection. The lower panel shows densitometry of blots. The error bars are mean $\pm$ SEM of three experiments. *p < 0.05 when compared with Adv-pgk. p < 0.05 versus GL with Adv-pgk. p < 0.05 versus DT with Adv-pgk.

One of the most common causes of acute tubular epithelial injury is ischemia/reperfusion and associated oxidative stress or ROS injury. Sensitivity to ischemia/reperfusion-induced injury differs along the renal nephron (27–

30). Apoptosis and necrosis were recorded previously in both PT and DT cell populations in ischemic renal injury (2,13,29). Therefore, correcting nephron dysfunction should be accomplished by transferring a particular

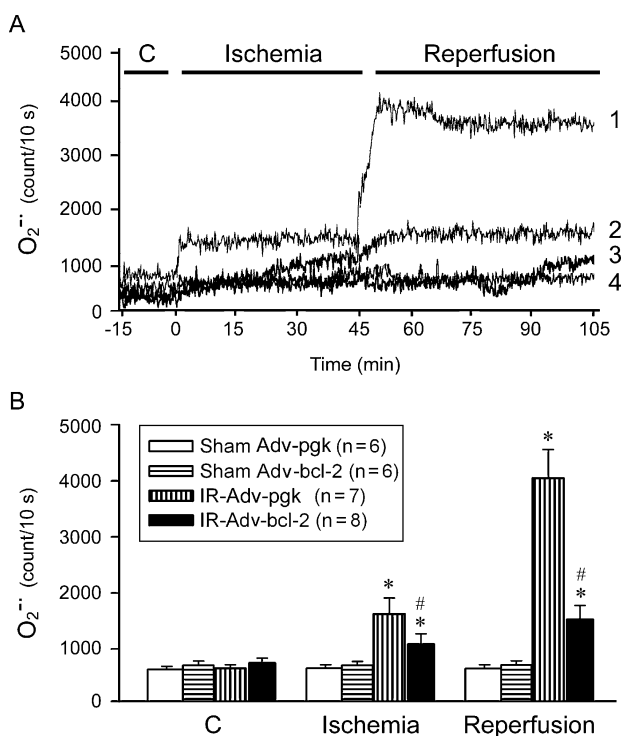


Figure 4: $O_2^{\cdot -}$ detection from rat kidney surface *in vivo*. (A) Typical recordings of MCLA (1.0 mM)-enhanced $O_2^{\cdot -}$ from the surface of ischemia/reperfusion kidney with Adv-pgk treatment (1, IR-Adv-pgk), ischemia/reperfusion kidney with pre-treatment of Adv-bcl-2 gene transfer (2, IR-Adv-bcl-2), sham-operated kidney with Adv-bcl-2 pre-treatment (3, Sham Adv-bcl-2) and sham-operated kidney with Adv-pgk treatment (4, Sham Adv-pgk). (B) Mean value of maximal $O_2^{\cdot -}$ amount detected from the kidney surface during the 15 min-control (C), 45 min-ischemia, and 4 h-reperfusion periods is displayed. Ischemia and reperfusion enhance $O_2^{\cdot -}$ production in the insulted kidney, however, the increased $O_2^{\cdot -}$ amounts are significantly attenuated by 7 days of Adv-bcl-2 pre-treatment. * $p < 0.05$ versus respective control stage. # $p < 0.05$ versus IR-Adv-pgk group.

molecule to the targeted nephron segments. In the current study, a specific tubular cell-targeted gene transfer techniques by a replication-defective Adv-bcl-2 could selectively transfect both PT and DT cells in the rat kidney without early local toxicity and $CD4^+/CD8^+$ -mediated immune response as assessed by renal histology and function (data not shown). Furthermore, we performed intrarenal arterial administration technique and 10 min of renal venous clamping, which resulted in bcl-2 transfer in the cortico-medullary area without renal damage.

Mitochondria are the target and source of ROS (31), which play an important role in physiologic signaling mechanisms and in regulation of apoptotic pathway (9,32). Mitochondrial dysfunction caused by inappropriate MTP opening disrupts mitochondrial membrane potential for ATP synthesis and triggers oxidative and anoxic cell death (33).

The outer mitochondrial voltage-dependent anion conductance (VDAC) channel is involved in cytochrome C release and is regulated by ROS and Bcl-2 family (16,34,35). $O_2^{\cdot -}$ but not H_2O_2 induces VDAC-dependent permeabilization of the outer mitochondrial membrane in HepG2 cells (34). Bcl-2 family are able to regulate the status of MTP; Bax, a channel-forming protein, can open it (9,36), and Bcl-2 and Bcl-xL are able to stabilize and inhibit its opening (37). Our data showed that Bax and $O_2^{\cdot -}$ are coexpressed in the ischemia/reperfusion kidney. Release of cytochrome C caused by increased MTP opening is a proximate trigger for evoking caspase 3 mediated apoptosis (8,16,35,36). Therefore, an increase in ROS and a reduction in Bcl-2/Bax ratio enhance MTP opening, cytochrome C release and caspase 3 mediated renal tubular apoptosis (9,32). According to our *in vitro* and *in vivo* data, the decreased ratio of Bcl-2/Bax by hypoxia/reoxygenation or ischemia/reperfusion injury triggers $O_2^{\cdot -}$ production, Bax translocation to mitochondria, cytochrome C release to cytoplasm, CPP32 activation and increases PARP fragments initiated apoptosis. Bcl-2/Bax ratio in mitochondria plays a critical role in regulation of apoptosis pathway.

Bonventre et al. (38) indicated that during 4–24 h of reperfusion, necrotic cell death of post-ischemic PT is initially more prominent than apoptotic cell death for a limited glycolytic ATP production (28), and a down-regulated bax and bak expression before injury (29). Iwata et al. (39) have found that PT injury was clearly discernible by 1 h of reperfusion, and overt tubular necrosis was first seen at 4 h of reperfusion, reaching its maximum 20 h later. Zager et al. (40) indicated that DNA fragmentation and tubular necrosis appeared simultaneously in the early (≤ 24 h) post-ischemic kidney, suggesting that it is not a primary mediator of ischemic cell death. Although TUNEL assay used in this study remains the most widely used technique, its specificity and sensitivity for the detection of apoptosis remain controversial. The specificity for apoptosis identification fell to 70% when a predominantly necrotic injury was induced (41). This may affect the identification of PT apoptosis or necrosis in the post-ischemic kidney. The DT was less sensitive to ischemia/reperfusion, with the dying cells being predominantly apoptotic (29,30). Anti-oxidant Bcl-2 resides in the mitochondria and prevents activation of the effector caspases by mechanisms such as blockade of MTP opening (16,37), by functioning as a docking protein (17), and by decreasing cellular ROS (15,19,42). However, with an *in situ* vascular NBT perfusion technique, our recent (2) and current data showed that the cellular source of ROS synthesis was mainly the PT epithelial cells in the ischemia/reperfusion kidney. The increased Bax expression, apoptotic cell death as well as the ROS production in PT cells can be ameliorated by superoxide dismutase (2) or Adv-bcl-2 administration. We also noted that, in culture, the number of apoptotic cells and the amounts of ROS formed are more evident in PT cells than in DT cells subjected to hypoxia/reoxygenation insult. Upon exposure to transient ischemia, the DT of the kidney often escapes

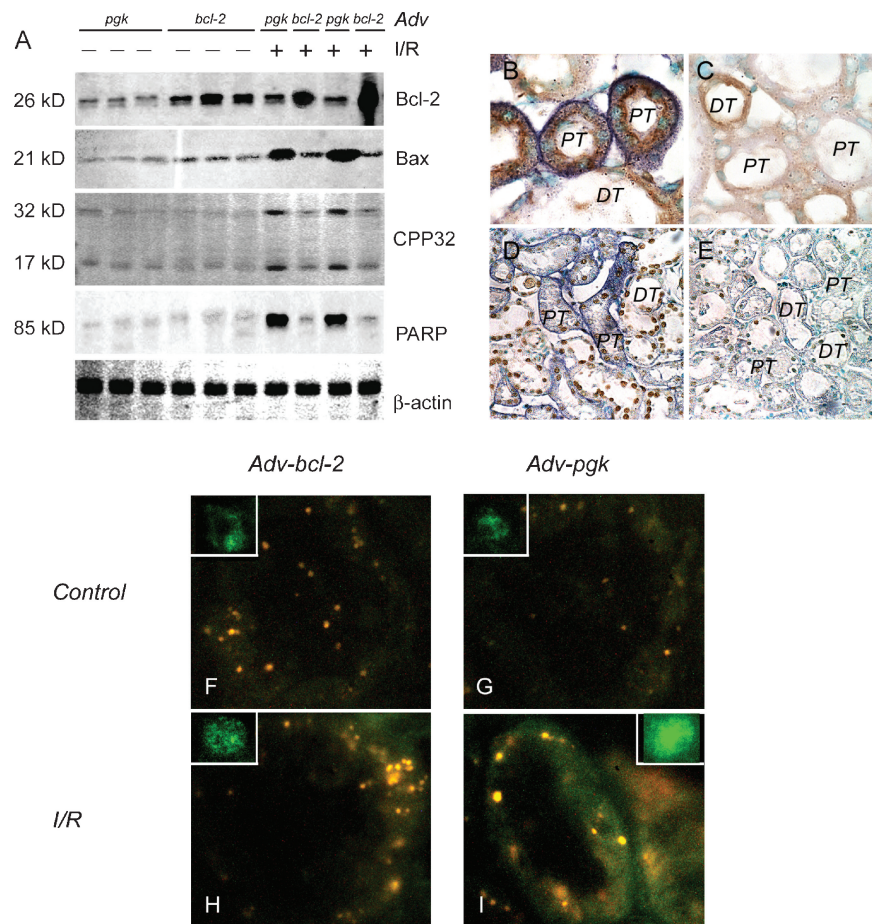


Figure 5: Effect of Adv-bcl-2 gene transfer on O_2^- , apoptosis-related proteins and apoptotic cell death of rat kidney subjected to ischemia/reperfusion injury. (A) In the baseline level, the Bcl-2 and Bax protein expression can be detected in the Adv-pgk kidneys ($n = 3$). Adv-bcl-2 gene transfer significantly enhances renal Bcl-2 protein expression ($n = 3$). In response to 45 min-ischemia/4 h-reperfusion (I/R), a mild increase in renal Bcl-2 protein and a marked enhancement in renal Bax protein are found to increase CPP32 and PARP expression. I/R enhanced Bax (brownish color) and O_2^- expression (blue deposits) is primarily expressed in the proximal (PT) but not in the distal tubules (DT) of Adv-pgk treated kidney (B). The decreased Bcl-2/Bax ratio by the I/R insult leads to O_2^- production and severe apoptosis (brownish nuclei) in the insulted kidney with Adv-pgk treatment ($n = 3$) (D). Adv-bcl-2 significantly decreases Bax and O_2^- coexpression in the I/R kidney (C) and consequently reduces apoptotic cells number in the Adv-bcl-2 treated kidney ($n = 3$) (E). In the control kidney with Adv-pgk (F) or Adv-bcl-2 (G) treatment ($n = 3$ each), a red-fluorescence heat shock protein 60 (HSP60) as a mitochondrial marker is highly stained in the tubular cells, however, a green fluorescence cytochrome C is not appeared in the tubular cytosol. I/R significantly increased green cytochrome C staining in the kidney with Adv-pgk treatment (I). Adv-bcl-2 treatment reduced the green cytochrome C staining (H). The insert of green color stain in the upper corner indicates the cytochrome C localization in the PT cell. I/R enhances green cytochrome C fluorescence in Adv-pgk treated PT (I), but not in Adv-bcl-2 treated PT (H).

the severe damage that afflicts the PT. The resistance to ischemia/reperfusion injury by DT cells may be attributed to increases of Bcl-2 protein expression and/or Bcl-2/Bax ratio in DT, whereas low levels of expression/ratio were detected in PT. This could also explain the generally low level of ROS formation in DT cells and Adv-bcl-2 treated kidney or cells. Over-expression of the Bcl-2, which largely localizes to the outer mitochondrial membrane, protects cells not only from apoptotic but also from oxidative cell death (42). Adv-bcl-2 administration significantly augmented Bcl-2 protein expression in DT and PT tubules *in vitro* and *in vivo*. Increased Bcl-2/Bax ratio by Bcl-2 augmentation

inhibited O_2^- production, cytochrome C translocation to cytoplasm, CPP32 activation and PARP fragments initiated apoptosis formation, but had no effect on Bax translocation to mitochondria. We implicate that augmented Bcl-2 protein by Adv-bcl-2 gene transfer confers renal DT and PT cells protection against hypoxia/reoxygenation or ischemia/reperfusion injury via the inhibition of MTP opening and cytochrome C release.

In summary, our results demonstrate that the rat kidney can be efficiently and selectively transduced with Adv-bcl-2 (10^8 pfu/kidney) without structural or functional side effect.

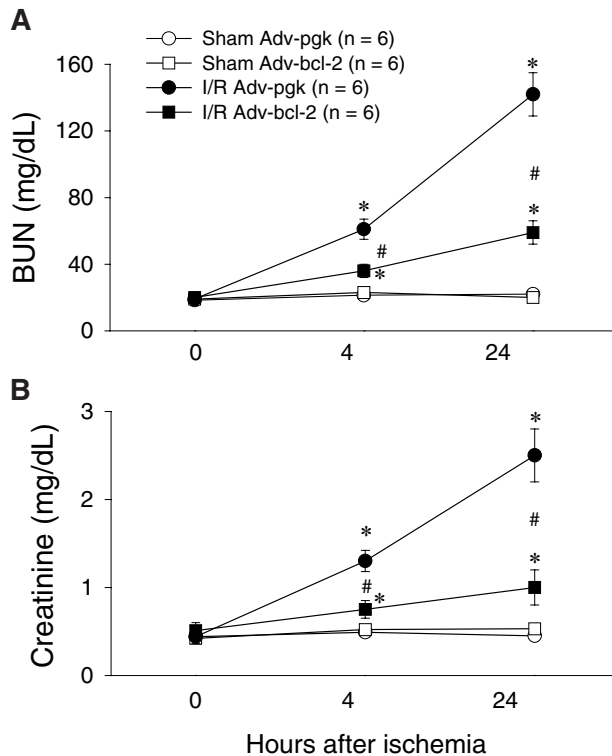


Figure 6: Effect of Adv-bcl-2 on blood urea nitrogen (BUN) and plasma creatinine (Creatinine) in the kidney with ischemia/reperfusion (I/R) injury. (A) BUN, (B) creatinine. In the Adv-bcl-2 group (n = 6), BUN and Creatinine levels at 4 and 24 h after ischemia were significantly lower than those of the Adv-pgk control group (n = 6). Data are expressed as mean $\pm$ SEM. *p < 0.05 versus their respective sham group, #p < 0.05 I/R Adv-bcl-2 versus I/R Adv-pgk group.

We provide evidence that gene delivery to the kidneys is feasible, and Bcl-2 augmentation in both PT and DT tubules protects against ischemia/reperfusion injury via the antioxidant and anti-apoptotic actions.

Acknowledgments

This work was supported partly by the National Science Council of the Republic of China (NSC 92-2320-B002-078, NSC 92-2314-B002-331 and NSC 92-2314-B002-163) and partly by the Institute of Biomedical Sciences Academic Sinica (IBMS-CRC92-T04).

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