Role of p21 and oxidative stress on renal resistance

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Abstract

Background. Subsequent ischemic episodes may induce renal resistance. P21 is a cell cycle inhibitor that may be induced by oxygen-free radicals and may have a protective effect in ischemic acute kidney injury (AKI). This study aimed at evaluating the role of oxidative stress and p21 on tubular resistance in a model of acquired resistance after renal ischemia and in isolated renal tubules.

Methods. Wistar rats were divided into: Group 1—sham; Group 2—sham operated and after 2 days submitted to 45-min ischemia; and Group 3—45-min ischemia followed after 2 days by a second 45-min ischemia. Plasma urea was evaluated on Days 0, 2 and 4. Serum creatinine, creatinine clearance and oxidants (thiobarbituric acid-reactive substances) were determined 48 h after the second procedure (Day 4). Histology, immunohistochemistry for lymphocytes (CD3), macrophages (ED1), proliferation (PCNA) and apoptosis (TUNEL) were also evaluated. Rat proximal tubules (PTs) were isolated by collagenase digestion and Percoll gradient from control rats and rats previously subjected to 35 min of ischemia. PTs were submitted to 15-min hypoxia followed by 45-min reoxygenation. Cell injury was assessed by lactate dehydrogenase release and hydroperoxide production (xenol orange).

Results. Ischemia induced AKI in Group 2 and 3 rats. Subsequent ischemia did not aggravate renal injury, demonstrating renal resistance (Group 3). Renal function recovery was similar in Group 2 and 3. Plasma and urine oxidants were similar among in Group 2 and 3. Histology disclosed acute tubular necrosis in Group 2 and 3. Lymphocyte infiltrates were similar among all groups whereas macrophages infiltrate was greater in Group 3. Cell proliferation was greater in Group 2 compared with

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Group 3. Apoptosis was similar in groups 2 and 3. The p21 expression was increased only in Group 3 whereas it was similar in groups 1 and 2. PTs from the ischaemia group were sensitive to hypoxia but resistant to reoxygenation injury which was followed by lower hydroperoxide production compared to control PT.

**Conclusion.** Renal resistance induced by ischaemia was associated with cell mechanism mediators involving oxidative stress and increased p21 expression.

**Keywords:** oxidative stress; p21; renal ischaemia; tubular resistance

**Introduction**

The main aetiology of acute kidney injury (AKI) is acute tubular necrosis mostly caused by prolonged renal ischaemia. Unfortunately, therapeutic strategies for AKI management are mostly supportive which have not effectively decreased mortality among these patients [1].

Tubular regeneration capacity, cell resistance and mechanisms of recovery post ischaemia have been studied because understanding these mechanisms could shed some light on AKI pathophysiology.

Acquired renal resistance after an ischaemic episode has been described [2–4]. Studies with isolated proximal tubules from animals submitted to previous ischaemia were resistant to hypoxia suggesting that cell factors are responsible for this resistance because extrinsic factors, such as inflammation and haemodynamic alterations, were excluded [5]. The role of inflammation in ischaemic AKI is not yet completely established, and its participation in ischaemic renal resistance has not been determined [6–9].

During tubular regeneration after AKI, remaining cells start the cell cycle for cell multiplication and renal tissue recovery. Cell cycle regulators expressed during this process are vital for an efficient tissue regeneration. Among these cell cycle regulators, p21 stands as a cycle inhibitor acting between G1 and S phases. This inhibitor has been largely studied in nephrotoxic and ischaemic models, and its protective effect has been demonstrated. It was demonstrated that the expression of p21 started 1 h after renal ischaemia, reaching its maximal expression at 24 h. Furthermore, immunohistochemical localization demonstrated p21 expression in the nuclei of tubular cells after ischaemia [10,11]. In cisplatin-induced DNA injury, presence of p21 may interrupt the cell cycle before duplication of defective genetic material. In addition, this protection may also be consequent to reduction of apoptosis due to inhibition of the caspase pathway [12,13].

In ischaemic AKI, p21 protection mechanisms are not determined. Apparently, p21 seems to be a cell survival factor after ischaemic injury, and its absence causes more severe AKI [14]. The P21 may be one of the renal resistance mediators because it was demonstrated that prior exposition to cisplatin induced p21 expression and kidney resistance to a second insult. This protection correlated with reduced apoptosis which could be mediated by an increased p21 expression [15]. Nevertheless, there are no studies addressing the role of p21 as a mediator of ischaemia-induced resistance.

This study aimed at evaluating the role of oxidative stress and p21 in ischaemia-induced tubular resistance in a model of acquired resistance after renal ischaemia and in isolated renal tubules.

**Material and methods**

**In vivo study**

Male Wistar rats (160–230 g) supplied by the University of Sao Paulo School of Medicine were divided into three groups: Group 1—sham-operated and after 48 h, another sham procedure was performed, Group 2—sham-operated and after 48 h, submitted to bilateral renal ischaemia for 45 min and Group 3—submitted to bilateral renal ischaemia for 45 min and after 48 h, another 45-min ischaemia, as described below:

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Sham</td>
<td>CrCl/histology</td>
</tr>
<tr>
<td>Group 2</td>
<td>Ischaemia</td>
<td>CrCl/histology</td>
</tr>
<tr>
<td>Group 3</td>
<td>Ischaemia</td>
<td>CrCl/histology</td>
</tr>
</tbody>
</table>

**Functional studies**

For functional evaluation, plasma urea determination was carried out (Urease Enzymatic Colorimetric Assay Kit, Labtest Diagnostics) at the beginning of the study (Day 0), 48 h after the first procedure (Day 2) and 48 h after the second procedure (Day 4). On Day 4, serum creatinine and creatinine clearance analysis were performed (Labtest Diagnostics). Evaluation was determined 48 h after the surgical procedures because we have previously observed that the peak urea occurred 48 h after ischaemia, recovering thereafter with plasma urea levels returning to baseline values on Day 4 (data not shown).

Subgroups from Group 2 (n = 4) and 3 (n = 4) were utilized for plasma urea determination on days 7 and 10 for evaluation of renal recovery.

**Determination of thiobarbituric acid reactive substances**

Rats (n = 5 for each group) were housed in metabolic cages without food or water before each clearance study, and blood and a 24-h urine sample were collected in order to determine plasma levels and urinary excretion of thiobarbituric acid reactive substances (TBARS). Samples were assessed using the thiobarbituric acid method, diluting a 0.2-mL sample in 0.8 mL of distilled water, to which 1 mL of 17.5% trichloroacetic acid was immediately added. Subsequently, 1 mL of 0.6% thiobarbituric acid, pH 2, was added. The sample was then placed in a boiling water bath for 15 min. After the sample had cooled, 1 mL of 70% trichloroacetic acid was added, and the mixture was allowed to incubate for 20 min. The sample was then centrifuged for 15 min at 2000 rpm. The optical density of the supernatant was read at 534 nm against a reagent blank using a spectrophotometer. The concentration of lipid peroxidation products was calculated as MDA equivalent using a molar extinction coefficient for the MDA-thiobarbituric acid complex of 1.56 × 10^5 mol−1 cm−1. TBARS were expressed as micromole per 24 h for urine sample or nanomoles TBARS per millilitre for plasma sample [16].

**Immunohistochemistry**

On Day 4, kidney tissue was harvested, fixed in Dubosque-Brasil solution, post-fixed in buffered formalin and embedded in paraffin. Kidney sections (3 μm) were analysed in haematoxylin-eosin and periodic acid–Schiff stains and immunoperoxidase method for the identification of T lymphocytes (CD3), macrophages (ED1) and cellular proliferation nuclear antigen (PCNA). Semi-quantification analyses were performed by a nephropathologist on each kidney slide obtained from each individual rat, in a blind mode regarding identification of rat group. For that purpose, immunohistochemistry positively marked cells were counted and
signed in 25 different high power fields using a Nikon microscope (with a ×40, ∞0, 17 WD 0.65 objective). Antigen retrieval protocol, primary antibodies, clones, sources, secondary antibodies and concentrations are summarized below:

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Antigen retrieval</th>
<th>Primary antibody titer</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal IgG-mouse anti rat CD68</td>
<td>ED1</td>
<td>Cedarlane (cod.MCA341R)</td>
<td>Trypsin enzyme</td>
<td>1:1600</td>
<td>EnVision peroxidase (Dako cod 1491)</td>
</tr>
<tr>
<td>Monoclonal IgG-anti CD3</td>
<td>E72.38</td>
<td>Dako (cod.M7254)</td>
<td>Steamer Tris/EDTA, pH 9.0</td>
<td>1:30</td>
<td>EnVision peroxidase (Dako cod 1491)</td>
</tr>
<tr>
<td>Monoclonal IgG-anti PCNA</td>
<td>PC10</td>
<td>Dako (cod M0879)</td>
<td>Microwave700w-citrate pH 6.0</td>
<td>1:1300</td>
<td>EnVision peroxidase (Dako cod 1491)</td>
</tr>
</tbody>
</table>

Staining was completed with chromogen 3,3′ diaminobenzidin (Sigma Chemical, CO, St Louis, USA), and sections were counterstained with haematoxylin [17–19].

**TUNEL**
The same semi-quantitative counting described above was used for apoptosis evaluation using the technique of deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL), which delineates single cells with fragmented DNA. Paraaffin sections were deparaffinized in xylene, rehydrated and rinsed in phosphate-buffered saline (PBS), pH = 7.4. Sections were treated with proteinase K (20 μg/mL) (Sigma Chemical, CO, St Louis, USA) for 15 min at room temperature. Sections were rinsed with PBS and incubated with terminal deoxynucleotidyl transferase (Apop Tag® Plus Peroxidase In situ Apoptosis Detection Kit, Chemicon International, Temecula, CA, USA) and Tdt enzyme 1:12 reaction buffer for 1 h at 37°C. Sections were incubated with anti-digoxigenin conjugate for 30 min at room temperature. Color development was achieved by incubation for 6 min with chromogen 3,3′ diaminobenzidin (Sigma Chemical, CO, St Louis, USA) and sections were counterstained with haematoxylin [20].

**Histology**
Paraaffin sections were stained with periodic acid-Schiff (PAS) and analysed by optical microscopy for the observation of structural alterations related to ischaemia and regeneration, mainly acute tubular necrosis.

**RT PCR (p21, p27 and b2 microglobulin)**
Total RNA was extracted by guanidinium thiocyanate–phenol–chloroform method (Trizol®, Invitrogen) [22]. The cDNA was synthesized from 2 μg of total RNA using an oligo dT primer (Promega, San Louis, EUA) and M-MLV-RT enzyme (Promega, San Louis, EUA). Polymerase chain reaction (PCR) was carried out to amplify the following specific cDNAs: p21, p27 and b2 microglobulin [21]. Magnesium chloride concentration, annealing temperature and number of cycles were adjusted for each pair of primers as shown below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>T°C annealing</th>
<th>N° cycles</th>
<th>MgCl (μl)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>b2 microglobulin</td>
<td>5′-CTCCCCAAATTCAAGTGTAATCTCTCTG-3′</td>
<td>55</td>
<td>25</td>
<td>1.5</td>
<td>249pb</td>
</tr>
<tr>
<td></td>
<td>5′-GAGTGGACGTTAATCTGCAAGC-3′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>5′-ATGTTCCGATCTGGTGTGATGTCGAGA-3′</td>
<td>60</td>
<td>35</td>
<td>2.0</td>
<td>499pb</td>
</tr>
<tr>
<td></td>
<td>5′-TCAGGGGTTCTCCTGCAAGA-3′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27</td>
<td>5′-GCACCTCAGCCGAGTTCTCTAC-3′</td>
<td>60</td>
<td>35</td>
<td>2.0</td>
<td>335pb</td>
</tr>
<tr>
<td></td>
<td>5′-TCTTTGGGGCTCTGTCAC-3′</td>
<td></td>
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</tr>
</tbody>
</table>

The PCR was performed mixing 2 μl of RT reaction product (cDNA), 10 μl of buffer (10x) (Tris-HCl 20 mM, pH 8.0; KCl 100 mM, EDTA 0.1 mM, DTT 1 mM, 50% glycerol, 0.5% 1μl de Taq DNA polymerase (5 U/μl, Promega, San Louis, EUA); and water up to 100 μl.

In **in vitro** study
Male Wistar rats (160–210 g) were submitted to 35-min ischaemia, and after 24 h, kidneys were removed for proximal tubules isolation (ischaemia group). Control tubules were isolated from normal rat kidneys.

**Proximal tubules isolation.** Proximal tubules were isolated according to methods previously described [23]. In brief, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) and underwent laparotomy for kidneys removal. Renal proximal tubules were isolated by collagenase digestion and separated by a Percoll gradient. Aliquots of 6 mL (containing 1.0–1.5 mg/mL of protein) were placed in ice-cold siliconized Erlenmeyer flasks for 5 min under 95% O2/5%CO2, followed by 10 min at room temperature.

**Hypoxia/Reoxygenation model**
After an equilibration period, isolated tubules were divided into an experimental group [hypoxia/reoxygenation (H/R)] and oxygenated group (control). Throughout the experiment, pO2 was maintained within the 200–300 mmHg range in control tubules. In H/R tubules, hypoxia (pO2: 20–40 mmHg) was induced by superfusing with 95% N2/5% CO2 for 5 min. Duration of hypoxia period was 15 min, and subsequently, tubules were reoxygenated by superfusing with 95% O2/5% CO2 for 5 min (pO2 returned to within the 200–300 mmHg range). Flasks were resealed, and tubules were maintained under reoxygenation for 45 min.

All samples were obtained at baseline, after hypoxia (at 15 min) and after reoxygenation (at 60 min).
**Table 1. Renal function evaluation (urea, creatinine—SCr and creatinine clearance—CrCl) on days 0, 2 and 4**

<table>
<thead>
<tr>
<th>Day 0 (n = 7)</th>
<th>Day 2 (n = 7)</th>
<th>Day 4 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urea (mg/dL)</strong></td>
<td><strong>Urea (mg/dL)</strong></td>
<td><strong>Urea (mg/dL)</strong></td>
</tr>
<tr>
<td>Group 1</td>
<td>35 ± 6</td>
<td>36 ± 7</td>
</tr>
<tr>
<td>Group 2</td>
<td>45 ± 12</td>
<td>46 ± 15</td>
</tr>
<tr>
<td>Group 3</td>
<td>44 ± 12</td>
<td>103 ± 40**</td>
</tr>
<tr>
<td><strong>SCr (mg/dL)</strong></td>
<td><strong>SCr (mg/dL)</strong></td>
<td><strong>SCr (mg/dL)</strong></td>
</tr>
<tr>
<td>Group 1</td>
<td>0.45 ± 0.1</td>
<td>0.55 ± 0.3</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.86 ± 0.2*</td>
<td>0.21 ± 0.1*</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.98 ± 0.1*</td>
<td>0.24 ± 0.1*</td>
</tr>
<tr>
<td><strong>CrCl (ml/min/100 g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. *P < 0.05 vs. Group 1; **P < 0.05 vs. Group 1 and 2; ***P < 0.05 vs. Day 0; ****P < 0.05 vs. Day 1 and Day 2.

**LDH release determination**

Cell injury was assessed by release of lactate dehydrogenase (LDH percentage). In order to determine LDH levels, 1 mL of tubule suspension was centrifuged for 2 minutes in a refrigerated centrifuge (1500 g) to separate supernatant and pellet. Pellet was lysed with Triton X-100. LDH activity was measured in supernatant and pellet according to Bergmeyer [24]. LDH activity was converted to percentage release by dividing supernatant activity by total activity.

**Hydroperoxide determination**

Hydroperoxide measurement: 1 mL of tubule suspension was obtained and sonicated immediately for 60 sec. Hydroperoxide was determined by xylenol orange method described by Jiang et al. [25]. Briefly, 100 μL of suspension was incubated for 15 minutes at room temperature together with 900 μL solution A sulphuric acid 25 mM diluted in methanol 90%, 100 μM of xylenol-orange (Sigma Chemical CO., St. Louis, USA), 4 mM de butyl hydroxitoluene (BHT, Sigma Chemical CO., St. Louis, USA), 250 μM FeSO4. Absorbance at 560 nm was measured immediately in a Hitachi spectrophotometer. Absorbance was corrected by extinction coefficient of butyl hydroperoxide 4.3 × 10^-4 M^-1 cm^-1. Values were expressed in micromole per milligram protein.

**Statistical analysis**

Data were initially submitted to K–S distance test to determine normality. Parametric data are presented as mean and standard deviation. Groups were compared with Student’s t-test for independent samples. For more than two samples, analysis of variance with repeated measures (ANOVA) was used with Student–Newman–Keuls post-test.

Interaction among different groups and times were compared by means of two-way ANOVA for non repeated measures with Student–Newman–Keuls post-test. Statistical significance was considered with *P ≤ 0.05.

**Results**

**Renal function**

On Day 2, plasma urea was significantly higher in Group 3, submitted to 45-min renal ischaemia on Day 0, compared with groups 1 and 2, sham operated on Day 0 (*P < 0.05). Plasma urea level on Day 4 was higher in Group 2 and 3, submitted to renal ischaemia on Day 2, compared with Group 1, sham-operated (*P < 0.05). There was no difference between groups 2 and 3 in addition, there was no a significant increase in plasma urea from Day 2 (second ischaemia) to Day 4 in Group 3 [103 ± 40 vs. 136 ± 44 mg/dL, not significant (NS)]. Similarly, serum creatinine levels and creatinine clearance were not different between groups 2 and 3 on Day 4 (Table 1).

Renal function recovery was similar between Group 2 and Group 3 as demonstrated by plasma urea on Day 7 (82 ± 25 vs. 83 ± 20 mg/dL, NS) and on Day 10 (53 ± 11 vs. 54 ± 11 mg/dL, NS).

Plasma and urinary TBARS measured on Day 4 were similar among all groups. Plasma TBARS for Group 1 was 7.4 ± 0.6 and for Group 2 and 3, 8.0 ± 0.4 and 10.6 ± 4.6 μmol/mL, respectively (all NS). Urinary excretion of TBARS was 294 ± 230 μmol/24 h for Group 1, which was not different from Group 2 and 3 (283 ± 181 and 538 ± 298 μmol/24 h, NS).

**Histology, immunohistochemistry and TUNEL**

Group 1 kidneys histology was normal in optical microscopy while animals from Group 2 and 3 presented similar acute tubular necrosis, characterized by interstitial oedema, tubular dilation with epithelial shedding and luminal debris, loss of brush border and nuclear cavirotosis. Group 3 animals showed marked regeneration with nuclear pleomorphism, mitosis and cardiomegaly (Figure 1).

Semi-quantitative lymphocyte count was similar among all three groups (7.3 ± 2.8 vs. 13.4 ± 9.2 vs. 8.6 ± 3.7 cel/mm², NS) as demonstrated in Figures 2A and 7A–C. In contrast, macrophages infiltrate was more intense in Group 3.
(23.5 ± 11.6 cel/mm², $P < 0.05$) compared with Group 1 (6.02 ± 1.9 cel/mm²) and Group 2 (10.42 ± 9.8 cel/mm²) as shown in Figures 2B and 7D–F.

The semi-quantitative analysis for cell proliferation, based on PCNA immunohistochemistry-marked tubular cells, was greater in groups 2 and 3 in comparison with Group 1 (Fig. 2A) and Group 2 Group 3

Fig. 2. Lymphocyte T (A) and macrophage (B) medullary Infiltrate. Semi-quantitative analysis of immunohistochemical staining with anti-CD3 (A) and anti-ED1 (B) antibodies on Day 4.

Fig. 3. Proliferating tubular cells (PCNA) (A) and tubular apoptosis (TUNEL) (B). Semi-quantitative analysis of immunohistochemical staining with anti-PCNA antibody (A) and TUNEL-positive cells (B) on Day 4.

Fig. 4. RT-PCR densitometric semiquantitative analysis (A) and representative gels of the amplified products (B), showing that p21 mRNA expression was greatly induced in Group 3 whereas Group 1 and 2 presented similar levels. No difference in p27 and $\beta_2$ microglobulin expression was found among groups.

Fig. 5. LDH release (percentage) in oxygenated and hypoxic/reoxygenated proximal renal tubules (PT) isolated from previous ischaemic kidneys (ischaemia) and control kidneys (control).
Group 1 (125 ± 28 cel/mm², P < 0.05). However, it was more intense in Group 2 (1262 ± 441 cel/mm²) than in Group 3 (653 ± 300 cel/mm², P < 0.05 vs. Group 2) as demonstrated in Figures 3A and 8A–C.

Apoptosis was similar between Group 2 and 3 (13 ± 2.7 vs. 15 ± 10.0 cel/mm², NS) but greater than Group 1 (2.5 ± 1.2 cel/mm², P < 0.05) as demonstrated in Figures 3B and 8D–F.

Expression of p21 mRNA
A significant increase in p21 mRNA expression was observed in Group 3 whereas groups 1 and 2 presented similar levels (Figure 4). No difference in p27 expression was found among the groups.

In vitro studies
LDH release (percentage) in oxygenated proximal tubules (PT) from control and ischaemia groups was similar at the beginning of the study. However, LDH release in oxygenated PT from kidneys of the ischaemia group was lower than in control group at 15 min (7.8 ± 1.7% vs. 11.7 ± 2.8%, P < 0.05) and at 60 min (10.9 ± 3.1% vs. 18.8 ± 4.1%, P < 0.05), suggesting that PT from kidneys subjected to prior ischaemia are more resistant to experimental time course injury. No difference was observed during hypoxia (15 min) between PT from control and ischaemia groups (34.1 ± 10.7% vs. 44.6 ± 10.9%, NS). In contrast, reoxygenation (45 min) caused lower LDH release in PT from ischaemia group compared with PT from control group (40.3 ± 8.9% vs. 55.4 ± 9.7%, P < 0.05) as shown in Figure 5, again indicating that PT from previous ischaemic kidneys are resistant to reoxygenation injury. In addition, PT from ischaemia group subjected to hypoxia/reoxygenation injury presented lower production of hydroperoxide compared with PT from control kidneys (1.0 ± 0.4 vs. 2.7 ± 0.3 μM/mg protein, P < 0.05) (Figure 6), suggesting that reduced oxidative stress may be related to the reoxygenation injury resistance in PT isolated from previous ischaemic kidneys.

Discussion
Injury and regeneration mechanisms are invariably triggered after ischaemia because intracellular signalling for both processes is related. In the present study, Group 3 rats were submitted to a second ischaemia during severe renal function impairment. Nevertheless, this second ischaemic episode did not aggravate previous acute kidney injury. In fact, Group 3 renal function, as demonstrated by serum creatinine, creatinine clearance and apoptosis were not different from Group 2, pointing to the development of renal resistance after an ischaemic injury. Furthermore, AKI recovery in Group 2 and 3 was similar on 7 and 10 days post-ischaemia.

Fig. 6. Hydroperoxide levels (microgram per milligram protein) in oxygenated and hypoxic/reoxygenated (60 min) proximal renal tubules (PT) isolated from previous ischaemic kidneys (ischaemia) and control kidneys (control).

Fig. 7. Representative pictures showing immunohistochemical staining with anti-CD3 (panels A, B and C) and anti-ED1 (panels D, E and F) antibodies of kidneys sections from Group 1 (A and D), Group 2 (B and E) and Group 3 (C and F) on Day 4.
Renal resistance occurs even when the second ischaemia is performed during the worse functional injury period. Zager et al. performed a second 40-min ischaemia 48 h after an initial ischaemia in rats. Functional evaluation demonstrated ischaemic resistance despite the animal’s previous reduced glomerular filtration rate [26]. However, mechanisms of ischaemic renal resistance remain unknown. Cochrane et al. reported that rats submitted to three 2-min ischaemic cycles followed by a 45-min ischaemia presented some protection compared with those submitted to ischaemia without previous ischaemic cycles. Such protection was not achieved when ischaemia was carried out by three ischaemic cycles of 5 min each [27]. In another study, Behrends et al. conducted experiments in pigs failing to report protection after three 10-min ischaemic cycles followed by a 60-min ischaemia [28].

Several studies indicate an early phase of post-ischaemic resistance obtained minutes or hours after brief ischaemic episodes (preconditioning), but whether this effect remains after longer time intervals is still controversial. Apparently, later resistance (after days or weeks) is better characterized after long periods of previous ischaemia [29,30]. Park et al. showed that mice submitted to 30-min ischaemia and further submitted to another 30- or 35-min ischaemia 8 days later, presented total protection against the second injury. However, if the initial ischaemia was 15-min duration, protection was partial, suggesting that protection was proportional to the initial injury [31].

After renal ischaemia, injured endothelial and tubular epithelial cells release chemotactic substances causing leucocytes infiltration and adherence. However, the role of different types of inflammatory cells, such as neutrophils, macrophages and lymphocytes in ARF has not yet been completely established, as well as their actual function during the repair process post-ischaemia [32]. In the present study, lymphocytes medullar infiltrate was similar among the three groups. No difference in lymphocytes infiltrate was found in Group 2 and 3, probably because of the timing of evaluation. Ysebaert et al. reported a significant increase in myeloperoxidase activity in the first 12-h post-ischaemia but without increased inflammatory cells infiltration. After 24 h, they have detected a marked increase in macrophages infiltrate, indicating diapedesis and tissue infiltration. Only in later phases (5–10 days), lymphocytes infiltrate became prominent followed by reduction in macrophages infiltration [33].

In contrast to lymphocyte infiltrate, it was observed a more intense macrophages infiltrate in Group 3. The role of these cells is still controversial in ischaemic ARF. However, there are suggestions that macrophages infiltrate might be associated with cell regenerative process. Persy et al. demonstrated that osteopontin knockout mice presented lower macrophage infiltrate during ischaemic AKI, but there was no functional benefit in these animals [34]. Activated macrophage are known to be capable of producing pro-inflammatory cytokines, oxygen-free radicals and myeloperoxidase. On the other hand, macrophages may play an important role as necrotic debris and apoptotic cell acceptors. In addition, activated macrophages are responsible for many re-epithelization growth factors, angiogenesis and turnover of extra cellular matrix, all vital requisites for tissue regeneration [35].

Balance between cell proliferation, necrosis, apoptosis and cell cycle coordination are vital for adequate tissue regeneration [11]. Park et al., using an ischaemic AKI experimental model, reported intense cell proliferation, mainly in S3 segment after 24 to 72 h of the first injury [36]. Later, an early induction of p21 expression after ischaemic AKI was reported. Increased oxygen-free radical production may be responsible for such induction, since this association has
been reported in some studies [37,38]. However, in the present study, different oxidant production could not be detected among groups, either in plasma or urine as assessed by TBARS. One reason for this discrepancy may be related to the late determination of these oxidant products, i.e. 48 h after renal ischaemia.

P21 protection mechanisms in ischaemic injury have not been completely determined but interruption of cell cycle is known to be a prerequisite for cell repair and regeneration to occur after ischaemia. Thus, cell cycle inhibition enables cell regeneration before its replication.

Megyesi et al. described an increase in p21 expression just 30 min after ischaemic clamp release [10]. However, such expression was not found at 48-h post ischaemia. In the present study, Group 1 and 2 rats did not present an increase in p21 expression. Only Group 3 rats presented an increase in p21 expression, suggesting its involvement as a renal resistance mediator post-ischaemia. PCNA immuno-histochemistry results are consistent with p21 expression findings, since there was reduced cell proliferation in Group 3 compared to Group 2.

Despite the two subsequent ischaemia episodes, Group 3 presented similar apoptosis degree compared with Group 2. In addition to cell cycle coordination failure, several studies have correlated the lack of p21 expression with more apoptosis. However, the mechanisms have not been established, and it seems that kinase proteins related to cell stress (JNK) may play a role in this process [12,13,39].

It is conceivable that the first ischaemic injury may have caused oxygen-free radicals synthesis which may stimulate p21 expression. During the second ischaemia, p21 protein overexpression could have possibly played a role as a renal resistance mediator. The increased p21 expression could be responsible for the reduced cell proliferation, the more intense apoptosis and an adequate cell cycle coordination, resulting in tubules resistance to reoxygenation injury.

Oxygenated PT from ischaemia group presented reduced time course cell injury compared with PT from control rats during the experiment, suggesting that these PT were more resistant independently of extrinsic factors. Furthermore, PTs isolated from kidneys submitted to prior ischaemia were sensitive to hypoxia but resistant to reoxygenation injury (Figure 5). One of the main mechanisms involved in reoxygenation injury is oxidative stress and consequent free radicals formation. In the present study, we have observed reoxygenation resistance followed by reduced hydroperoxide production in PT from ischaemia group, suggesting that protection against reoxygenation injury may be partially attributed to the lower synthesis of oxygen-free radicals. Zager et al. [40] showed that PT isolated from rats submitted to prior ischaemia presented lower malondialdehyde production after hydrogen peroxide challenge in comparison with tubules from normal rats, suggesting oxidative stress resistance.

In conclusion, renal resistance obtained by ischaemia was associated with cell mechanisms involving oxidative stress and increased p21 expression as mediators of this protection which correlated with reduced cell proliferation and the occurrence of same degree of apoptosis.

Conflict of interest statement. None declared.

References

Sulodexide ameliorates early but not late kidney disease in models of radiation nephropathy and diabetic nephropathy

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Abstract

Background. Sulodexide is a glycosaminoglycan with anticoagulant and antithrombotic activities. Although sulodexide reduced albuminuria in patients with type 1 and type 2 diabetes, long-term effects on chronic renal injury are not established. We investigated sulodexide effects and mechanisms in a rat radiation nephropathy model and in the db/db mouse model of diabetic kidney disease.

Methods. Sprague–Dawley rats received kidney radiation and were treated as follows: 15 mg/kg/day sulodexide s.c., 6 day/week (SUL) or no treatment (CONT). Subsets of animals were sacrificed after 8 weeks and 12 weeks. Blood pressure, serum creatinine, creatinine clearance (CrCl) and urinary protein excretion were measured every 4 weeks. Sclerosis and plasminogen activator inhibitor-1 (PAI-1) expression were assessed at 8 and 12 weeks, and collagen I, total collagen content and phospho-smad-2 expressions were determined at 12 weeks. Twelve-week-old db/db mice received sulodexide as above or vehicle. Albuminuria and CrCl were assessed at intervals till sacrifice at week 9 with assessment of urinary transforming growth factor-β (TGF-β) and glomerular lesions.

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