Apoptosis and necrosis during ischaemia in renal tubular cells (LLC-PK₁, and MDCK)

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Abstract

Background. Ischaemia is the most frequent cause of acute renal failure. It has been previously demonstrated that ischaemia is connected with signs of necrosis and apoptosis. Apoptosis is an energy-dependent process. During ischaemia intercellular energy levels decline rapidly. Therefore, the goal of the investigation was to reveal the time dependency of cell death mechanisms during ischaemia leading to irreversibility of tissue damage.

Methods and results. A model of renal ischaemia induced by ATP depletion was used in LLC-PK₁ and MDCK-cells. Cell proliferation, determined by ³H-thymidine and BrdU incorporation and by the Ki67-labelling index was affected already after 1–2 h of ATP depletion in both cell lines. Cell viability and membrane leakage, estimated by trypan blue and propidium iodide exclusion and LDH release, was profoundly increased after 8–16 h. Evaluation of mechanisms of necrotic or apoptotic cell death was calculated from fraction of cells with pyknotic nuclei, investigation of DNA fragmentation and, by translocation of phosphatidylserine (PS) from the inner membrane face to the surface. In both cell lines increased numbers of cells with condensed nuclei was not a major sign of apoptosis. Only in MDCK cells were the numbers of cells with condensed nuclei significantly increased after 1 h compared to controls. As a hallmark of apoptosis, ATP depletion resulted in intranucleosomal DNA fragmentation after 1 h. After 8–16 h this pattern changed to a smear pattern, as a sign for necrosis. PS staining was detectable at the cell surface after 1 h.

Conclusions. Ischaemia is associated with a rapid loss of proliferation and signs of apoptosis at early stages in a small proportion of cells. Most cells undergo the necrotic pathway of cell death after prolonged ATP depletion (8 h). There was no difference in behaviour comparing proximal (LLC-PK₁) with more distal (MDCK) cell culture models. These results may help to explain the findings that apoptosis and necrosis have both been described after renal ischaemia.

Key words: ATP depletion; cell proliferation; acute renal failure; cell death

Introduction

Renal tubular cells are morphologically and functionally specialized epithelial cells for transport of ions, water, and macromolecules across cell layers. Reabsorption and secretion by tubular cells are selective and energy-dependent processes. These processes are perturbed by ischaemic injury. ATP depletion has been introduced to study ischaemia in cell-culture models. It has been shown before that ATP depletion in renal cells affects the cytoskeleton [1–8], tight-junctions [9], enzymes [2,10], transport processes [11], and the transcription of genes [12]. Cell death is the eventual end-point of this injury. Two types of cell death with different morphological and biochemical changes have to be considered: necrosis and apoptosis. Classical necrosis is a passive process, beginning with the loss of homeostatic processes, cell swelling, and mitochondrial changes, and ending in cell lysis. The DNA is preserved initially and is non-specifically disintegrated later. Apoptosis is an active, energy-requiring process that differs from necrosis. Cells undergoing apoptosis are characterized by nuclear chromatin condensation and morphological changes that proceed in the absence of membrane leakage. The DNA is damaged and broken into distinct mono- and oligosome fragments by endonuclease activity [13–15].

The mechanism of cell death initiated by an ischaemic impact is the key feature for the possibility of recovery. Both cell death mechanisms, necrosis or apoptosis, have been described during ischaemia [16–21]. Whereas the passive necrotic pathway of cell death is not reversible, the active apoptotic way is potentially preventable. During ATP depletion energy levels rapidly decline [25]. Since apoptosis is an energy-dependent process it is likely that cell death mechanisms during ischaemia are a function of time. The purpose of this study was therefore to evaluate the time course of inhibition of proliferation and cell death during ATP depletion. Damage after renal failure is
not uniform in all cell types [22,23]; thus, we examined
the importance of ischaemia in renal tubular cells from
two different parts of the nephron. LLC-PK₁-cells were
used as a model of the proximal tubule and MDCK-
cells as a model of the distal tubule/collection duct
respectively.

Materials and methods

Cell cultures

LLC-PK₁-cells, passages 177–186, and MDCK-cells, pas-
sages 69–77, were cultured as described before [24]. In brief,
cells were grown under standard conditions in Dulbecco’s
modified Eagle’s medium (DMEM) supplemented with 10%
fetal calf serum, 5 mmol glucose, 4 mmol glutamine, 10 mmol
HEPES and 100 U/100 ng/ml penicillin/streptomycin in a
humidified atmosphere of 5% CO₂/95% air at 37°C.

For the [³H]thymidine studies cells were subcultured in
6-well plates (Costar®) and in 96-well trays (NUNC) for
BrdU studies. For Ki67, haematoxylin and annexin studies
they were seeded on glass slides. For trypan blue, LDH, and
DNA isolation cells were grown in 6-well plates (Costar®).
Cells were used for this experiments when a confluence of
about 75–80% was reached.

Cell culture medium, serum, and supplements were
obtained from Boehringer–Mannheim, Germany.

ATP depletion

Monolayers were washed twice with PBS and incubated for
3 h in a glucose-free preincubation medium (125 mmol NaCl,
5 mmol KH₂PO₄, 2 mmol MgSO₄, 25 mmol NaHCO₃,
1.5 mmol CaCl₂, 2 mmol glutamine) to reduce endogenous
glycolytic substrates. After that, cells were incubated with
depletion medium (same as preincubation medium, but with
10 μmol antimycin A, and 10 mmol 2-deoxyglucose, without
glutamine) according to the method of Doctor et al. [25]
with the difference, that antimycin A was used instead of
rotenone. Antimycin A and 2-deoxyglucose were purchased
from Sigma; all other chemicals were purchased from Merck
if not specifically indicated.

For determination of newly synthesized DNA ([³H]thymidine,
BrdU) and the Ki67 labelling index, cells were ATP depleted
for 1, 2, 3 and 4 h. For all other studies measurements at 8, 16, 24, 48 and 72 h were performed in
addition. Cells for controls were grown simultaneously in
DMEM.

Determination of cell proliferation

[³H]thymidine incorporation. After ATP depletion, medium
was removed and cells were washed three times with PBS;
DMEM (without FCS) containing 1 mCi/ml [³H]thymidine
(Amersham) was used for pulsing for 1 h at 37°C. The
radioactive thymidine medium was removed after this time
and replaced by 2 mmol cold thymidine (Sigma) DMEM
medium to reduce further [³H]thymidine incorporation by
competition. Cells were incubated for 3 h in this medium.
Lysis of cells was done with a lysis buffer (1% Triton X–100,
25 mmol glycerol, pH 7.8), 15 mmol MgCl₂, 4 mmol
EGTA, 1 mmol dithiothreitol; 300 μl lystate was given to
2.7 ml scintillation liquid. Radioactivity was detected by a
Tri-Carb 1900TR liquid scintillation analyser (Packard
Instruments). Duplicate determinations were carried out.
The background was determined by measuring four vials of
pure lysis buffers in the scintillation liquid. The mean value
of the four background determinations was calculated and
subtracted from the mean of the duplicate determination
value of each experiment.

BrdU incorporation. The BrdU Labeling and Detection
kit (Cell Proliferation ELISA BrdU, colorimetric,
Boehringer–Mannheim, #1647229) was used. Cells were
washed three times with PBS and incubated in BrdU solution
(dilution 1:100 in DMEM without FCS) for 6 h at 37°C.

After removal of BrdU solution cells were fixed and dena-
tured with the ready-for-use FixDenat solution for 30 min.

Then cells were incubated with anti-BrdU-POD working
solution (dilution 1:1,000 in the ready-for-use antibody
solution) for 90 min. After that the antibody working solution
was decanted and cells were washed three times with the
washing solution. The substrate was added and incubated
until a blue colour was obtained. The addition of 1 mol
H₂SO₄ stopped the reaction. BrdU incorporation was meas-
ured with a Titertec ELISA Reader MCC/340 at 450 nm.
The blank row was established by omitting BrdU solution
from one experimental row of the 96-well tray. For every
experimental row (=12 wells) of the 96-well tray the mean
was determined and the mean of the blanks subtracted.
These studies were carried out in parallel to the
[³H]thymidine experiments.

Ki67 labelling. After ATP depletion cells were fixed in 4%
formaldehyde. The fixation fluid was removed by washing
with ethanol. Cells were rehydrated and incubated for 30 min
with 3% H₂O₂. Slides were placed in citrate buffer and boiled
for 6 min under pressure. After cooling 10% bovine serum
albumin was added for 10 min. Then the cells were incubated
for 1 h with MIB-1 monoclonal mouse antibodies
(Calbiochem #NA21–2, dilution 1:100) and washed with
PBS. The second biotinylated anti-mouse antibody was added
for 45 min. Immunoreaction was followed using horseradish-
peroxidase-labelled avidin–bixin-complex (ABC) for 30 min
devolved with diaminobenzidine. Nuclear counterstain-
ing was carried out with haematoxylin for 1 min. Cells were
considered Ki67 positive when brown nuclear staining could
with the di

LDH release

LDH release was determined by a photometric assay (Sigma).
Fifty microlitres of the centrifuged cell supernatant was
incubated for 1 h at 37°C with 1 ml pyruvate substrate.
Extinction was measured at 380 nm [24].
Determination of condensed nuclei

Cell culture, ATP depletion, and cell fixation was carried out as described above. Cells were stained for 15–20 min in haematoyxlin solution, dehydrated in increasing concentrations of ethanol, finally treated in xylol, and embedded. The number of condensed cells in the total cell number was counted randomly for 10 high-power fields for each slide.

Fas-induced apoptosis in Jurkat cells (positive control for apoptosis [27])

In Jurkat cells, a T-lymphoblastoid cell line, apoptosis was induced by incubation with 100 ng/ml anti-human Fas IgM (clone CH-11, Upstate Biotechnology) overnight.

Detection of DNA fragmentation by agarose gel electrophoresis

Endonuclease activity was evaluated by detection of mono- and oligonucleosome DNA fragments as a strong marker for apoptosis. DNA was isolated from LLC-PK₁ and MDCK as previously described by Hagar et al. [19,20] with modifications. Cells were collected by centrifugation and lysed in 0.5% Triton X–100, 10 mmol Tris-HCl, pH 8.0, 25 mmol EDTA for 1 h at 4 °C. The lysates were incubated overnight with proteinase K (20 mg/ml) at 50 °C. RNase A (1 mg/ml) was added and incubated for 4 h. After ethanol precipitation the DNA was redissolved in water. In each sample the concentration of DNA was determined by UV absorption spectrophotometry at 260 nm. The same amount of DNA from each sample was separated by electrophoresis through a 2% agarose gel and visualized after ethidium bromide staining by UV fluorescence.

Detection of phosphatidylserine (PS)—Annexin method [28]

Detection of PS was done using an incubation buffer (10 mmol HEPES/NaOH, pH 7.4, 140 mmol NaCl, 5 mmol CaCl₂) containing annexin-V-fluos (Boehringer–Mannheim) at a final concentration of 1 µg/ml. Propidium iodide (1 µg/ml) was added to distinguish between cells undergoing necrosis and those undergoing apoptosis. Cells were incubated for 10 min in this solution and examined by fluorescence microscopy (Olympus BX50).

Presentation of data and statistics

Data are shown in percentage as the mean ± SEM of the DMEM time-control experiments. Significance was determined by comparison of the data of each test group vs the control group using the independent-samples t test with SPSS 7.0 for Windows. Significant levels were set at \( P \leq 0.05 \).

Results

DNA-synthesis in LLC-PK₁ and MDCK-cells

In LLC-PK₁-cells DNA synthesis, monitored by \(^{3}H\) thymidine and BrdU incorporation, was decreased significantly after 1 h. Using the BrdU method, proliferation was reduced after 1 h of ATP depletion to 45.3 ± 19.9%, compared to controls. In the course of 4 h, DNA synthesis was further reduced to 23.3 ± 14.4% of controls (Figure 1a). The determination of DNA synthesis with \(^{3}H\) thymidine incorporation demonstrated similar results. After 1 h of ATP depletion the counts decreased to 20.8 ± 11.5% of controls and were reduced further after 4 h to 12.5 ± 8.9% (Figure 1a).

In MDCK cells after 1 h of ATP depletion, DNA synthesis, shown by BrdU incorporation, was lowered significantly to 75.6 ± 14.2% of controls. By 4 h of ATP depletion DNA synthesis decreased to 31.3 ± 18.2% (Figure 1b). Using \(^{3}H\) thymidine incorporation, as an indicator, the DNA synthesis was reduced significantly to 25.6 ± 11.5% after 1 h and dropped to 5.9 ± 1.7% after 4 h (Figure 1b).

Results of both methods underscore that the DNA synthesis was decreased by ATP depletion. However,
the responses for the [3H]thymidine and BrdU methods were different. Nevertheless, the data for the [3H]thymidine method and the BrdU method showed a high degree of comparability. The correlation coefficient (Spearman’s rho bivariate correlation) for the two techniques is 0.93 for LLC-PK1-cells and 0.95 for MDCK-cells.

**Ki67 labelling index**

To study the impact on cell-cycle-specific proteins, the expression of the nuclear antigen Ki67 was evaluated [29]. Ki67 is exclusively expressed during the G1, S, G2 and M phases but not in resting cells (G0) [29–33]. In LLC-PK1-cells, the Ki67 labelling index in ATP depleted cells was significantly decreased after 2 h compared to controls and was further significantly decreased at 3 and 4 h of ATP depletion (Figure 2a). In MDCK cells, the Ki67 labelling index dropped significantly after 3 h compared to controls (Figure 2b). A representative example for evaluation of Ki67 labelling by MIB is given in Figure 3a for MDCK cells grown 4 h in DMEM, as control. Figure 3b shows a part of a high-power field after 4 h ATP depletion with a reduced number of Ki67-positive cells.

For longer ATP-depletion studies, the Ki67 labelling index for adherent cells like LLC-PK1 and MDCK cells growing on glass plates was not feasible, because cells tended to lift off from the slide after prolonged ATP depletion.

**Trypan blue exclusion**

To distinguish between viable and irreversibly damaged cells, the ability of cells to exclude trypan blue was used [26]. In both cell lines, the amount of non-viable cells was not significantly increased after the first 8 h of ATP depletion. After 16 h ATP depletion, 54 ± 6.9% of LLC-PK1 cells and 36.9 ± 5.2% of MDCK cells were irreversibly damaged (Figure 4), with an increase to 100% of damaged cells during prolonged ATP depletion for up to 72 h.
Fig. 4. Determination of viable cells by trypan blue exclusion: During the first 8 h of ATP depletion the numbers of trypan-blue-positive cells was not significantly increased. Significant loss of ability to exclude trypan blue was apparent after 16 h of ATP depletion. Data are given as mean ± SEM (*P ≤ 0.05).

LDH release

We examined the effect of ATP depletion on LDH release, as a marker for the cytoplasmatic compartment. LDH was not significantly increased during the first 8 h of ATP depletion. After 16 and up to 72 h, LDH was increased significantly in LLC-PK₁-cells and after 24 to 72 h in MDCK-cells (Figure 5) compared to controls.

Haematoxylin labelling index [34]

In LLC-PK₁-cells the level of condensed nuclei was not significantly increased during ATP depletion over 4 h (Figure 6a) compared to controls, but significant compared to 0 h controls after 3 h of ATP depletion. In MDCK cells the level of condensed nuclei was significantly increased after 1 and 3 h of ATP depletion (Figure 6b), compared to controls. Compared to the 0 h value, a significantly increased level was observed after 1–4 h of ATP depletion.

A representative example for part of a high-power field with condensed nuclei of MDCK-cells after 4 h of ATP depletion is shown in Figure 7b. As control, Figure 7a shows a part of a high-power field with MDCK cells grown in DMEM. For Ki67 studies longer times of ATP depletion were not feasible, because cells lost adherence to the slides.

Detection of DNA fragmentation by agarose gel electrophoresis

DNA fragmentation was used as the strongest marker for apoptosis, since laddering is found almost universally in apoptotic cells. In Jurkat cells, as a positive
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Translocation of PS and membrane permeability for propidium iodide

Translocation of PS from the inner membrane face to the membrane surface is an early event of cell death [28]. To differentiate between apoptosis and necrosis, propidium iodide was used to detect necrotic cells [28]. PS was detectable in many cells after 1 h of ATP depletion at the membrane surface in both cell lines. Only single cells were propidium iodide positive after this time. This observation held true during the first 4 h of ATP depletion. At 8 h of ATP depletion, many but not all cells were propidium iodide positive, whereas after 16 h all cells were propidium iodide positive. Figure 9 shows ATP-depleted cells from 1 to 16 h (left panel LLC-PK\(_1\) cells, right panel MDCK cells). After 1–4 h PS was detectable all over the cell surface. The majority, but not all, cells were permeable for propidium iodide after 8 h. After 16 h all cells were propidium iodide positive.

Discussion

The occurrence of cell death has been shown in several renal diseases and in previous in vivo and in vitro studies [16–20,40–42]. In the present study it could be shown that ATP depletion in LLC-PK\(_1\) and MDCK-cells is initially associated with a rapid loss of proliferation and with signs of apoptosis by different methods, whereas prolonged ischaemia is associated with signs of necrosis. The activation of programmed cell death by ATP depletion in a share of cells and final cell death by necrosis of most after prolonged ATP depletion regarding the time scale has not been shown before for LLC-PK\(_1\) and MDCK-cells in vitro.

Cell proliferation

Measuring proliferation by detection of newly synthesized DNA via incorporation of a precursor, using the \(^{3}H\)thymidine or the BrdU methods, is dependent on after 0, 1, 2, 3, 4, 8, 16, 24, 48 and 72 h of ATP depletion. In LLC-PK\(_1\) cells apoptotic DNA laddering (lane 20–16) is visible after 1 h of ATP depletion changing to a 'smeary' pattern between 8 and 16 h (lane 15/16) as a sign of necrosis. In MDCK cells DNA laddering is clearly visible during 1–8 or 16 h of ATP depletion (lane 10–6/5, changing to unspecific necrotic decay between 16 and 24 h ATP depletion (lane 6 and 5).
Fig. 9. Detection of phosphatidylserine (PS) and membrane permeability for propidium iodide in LLC-PK$_1$ (left panel) and MDCK cells (right panel). In controls at time 0, PS molecules are not detectable at the membrane surface. After 1–4 h of ATP depletion PS molecules are translocated to the membrane surface (arrows) and are detectable with fluorescence-labelled annexin. After 8 h of ATP depletion, membranes of many, but not all, cells are permeable to propidium iodide. All cells were propidium iodide positive after 16 h as a sign of necrosis.
of a DNA precursor after ATP depletion may produce erroneous results because the metabolic inhibitors antimycin A and 2-deoxyglucose remain within the cells after removal of the depletion medium. Thus the real reduction in proliferation may be overestimated by these methods. However, for the proliferation studies determining the expression of Ki67, cells were fixed directly after ATP depletion. Because of that, the metabolic inhibitors were not longer physiologically active and the problem of continued inhibitor activity during incorporation of DNA precursors was bypassed in these experiments. The Ki67 experiments showed comparable results to the experiments using a labelled DNA precursor to determine cellular proliferation. Therefore it can be concluded that proliferation is significantly inhibited by ATP depletion.

ATP levels rapidly decline to a low content by metabolic respiration chain inhibitors like antimycin A in combination with glycolytic not usable substrates like 2-deoxyglucose as shown by others [6–8]. As seen in Figures 1a and 1b the DNA synthesis decreased significantly after 1 h ATP depletion and fell further in the course of 4 h to about 10% of the controls. Since cellular proliferation is strongly energy dependent these results indirectly reflect the drop in intracellular ATP levels during the ischaemic impact.

During the first 2 h of ATP depletion, proliferation in MDCK-cells was less affected than in LLC-PK1-cells. This corresponds well with the observation in humans, that ischaemic renal failure damage is predominantly localized in the proximal tubule, at least in the early phase of an ischaemic injury [23]. At 3–4 h, MDCK cells were more vulnerable than LLC-PK1 cells. This observation supports the clinical experience that prolonged ischaemic renal failure is associated with additional distal tubular disturbance [23].

**Apoptosis/necrosis**

Apoptosis and necrosis are the two kinds of cell death. Whereas the necrotic form of cell death is a passive act, apoptosis is an energy-dependent process. Several features are described to differentiate between the two forms of cell death [13]. Different methods were developed and published to recognize the different features of cell death. Unfortunately the results of these methods are not always unequivocal. Furthermore not every described hallmark for apoptosis is a general feature for this type of cell death. Thus it is recommended that independent methods are utilized to decide between apoptotic or necrotic cell death, using the sum of the obtained signs.

**DNA laddering**

Apoptosis is activated initially by ATP depletion early after the initiation of ischaemia, and prolonged ATP depletion resulted in necrosis as predominant feature. This is supported by the appearance of a typical electrophoretic DNA ladder. This intranucleosomal DNA fragmentation by endonuclease activation has been considered as one key feature of apoptosis [15,27]. As seen in Figure 6 the DNA laddering pattern changed to a smearly pattern, as a sign of necrotic cell death between 8 and 16 h of ATP depletion.

**Chromatin condensation**

Each dye used to detect nuclear chromatin alterations during cell death as an apoptotic sign has advantages and disadvantages. Haematoxylin staining has been shown to be a useful tool in several studies to detect pyknotic nuclei [34,35]. In our study the number of cells with condensed nuclei was not significantly increased in LLC-PK1 cells during 4-h ATP depletion compared to controls, but was significantly increased compared to 0 h. Compared to controls in MDCK cells the number of cells with pyknotic nuclei was significantly increased, but the effect was small. This indicates that apoptotic cell death mechanisms were activated in a minority of cells during the early ischaemic impact or that nuclear condensation is not a key feature of apoptosis in the two cell lines used.

**Annexin method**

Translocation of PS from the inner face of the membrane to the cell surface is one of the earliest events of cell death [36,37]. This shifting of PS has been described as a fundamental feature of cell death [38]. Since exposition of PS at the external cell surface is not only an apoptotic but also a necrotic feature, the combination of the annexin method with the use of chromatin dyes such as propidium iodide can simultaneously identify and distinguish between the two types of cell death. Only the membranes of necrotic cells are permeable to propidium iodide. As seen in Figure 9, PS was detectable already after 1 h on the surface of several but not all cell membranes, whereas the nuclei were not stained by propidium iodide. Because the dye-exclusion ability for propidium iodide is still intact, these cells with translocated PS molecules were considered to be undergoing apoptosis not necrosis.

Counts of the absolute number of cells with translocated PS at the membrane surface, as well as the number of cells with membranes permeable to propidium iodide, were not possible by fluorescence microscopy as the annexin method was used to monitor cell death in living, unfixed cells. Slides dry out and the fluorescence fades during observation. As described and shown in Figure 9, PS translocation was already visible after 1 h ATP depletion in some, but not in the majority, of treated cells. In Figure 9 only small areas of the monolayers are shown, suggesting the presence of a higher share of apoptotic cells detected by the annexin method compared to the haematoxylin method. However, these results are qualitative and should be taken as further evidence for apoptosis and not necrosis as an early event in affected cells during ATP depletion.

Flow cytometry for quantitative determination of
PS translocation, cannot be performed in monolayers, only in single-cell suspension. Since preservation of cell–cell interaction is one of the features of cell damage, flow cytometry was not used in this study.

Our results may help to explain the variability between the results of other studies showing apoptosis or necrosis after ischaemia [13–21]. The physiological function of the initial activation of the apoptotic pathway is perhaps part of an emergency programme to preserve the cell barrier and the tubular function.

As described by others [42], renal tubular cells produce an array of substances during an ischaemic renal insult. The release of substances like cytokines may be one mechanism to protect affected cells against further damage during an ischaemic impact, and to prepare the cells for recovery. Ischaemic cells may be sensitive for auto- or paracrine acting survival factors and cell death may be influenced by regulatory stimuli. For the clinical importance the ischaemic impact would be preventable or by application of such substances if the ischaemic impact could be predicted.

Enhanced cell survival by mobilization of heat stress proteins during ATP depletion was observed in proximal tubule cells [43]. Thus the rapid fall in DNA synthesis may be a consequence of apoptotic DNA damage and a physiological ‘sorting out’ of affected cells.

According to the classic necrosis model [13,17] the impact of prolonged ATP depletion is non-specific and the whole cellular metabolism is decreased after consumption of cellular ATP stores. Signs of cell death change from apoptosis in the initial phase after the ischaemic injury to necrosis after prolonged ATP depletion. However, loss of cellular energy for 8 to 16 h is a long period. Our results lead to the speculation that cell death during the early phases of ischaemia may be inhabitable.

Clinical relevance

Cell culture models of ATP depletion do not necessarily reflect the issues in human acute renal failure, and results obtained in cell cultures cannot be transferred directly onto the human situation. However, ATP depletion mimics at least some of the features of acute renal failure, like disruption of the cell–cell interaction, loss of cell polarity etc., and the cell culture models helped tremendously in the understanding of cellular actions and mechanisms [1–13,23,25,41,43]. In that respect the present work may help in the understanding of the findings, during acute renal failure, of apoptosis and necrosis at the same time. Furthermore, these results lead to the hypothesis that apoptosis is present only as long as cellular energy levels are above a critical level and that necrosis is a feature of long-lasting hypoxia. Since apoptosis is at least theoretically inhabitable, the initial phase after renal injury may be preventable.

In conclusion, our results indicate that the apoptotic pathway of cell death seen at the early phase of ATP depletion is activated in only a small fraction of cells, whereas the bulk of cells die by necrosis during persistent ischaemia in the two cell lines used [39]. The cells in culture representing parts of the proximal and distal tubules were affected to the same extent.

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