EFFET DE L'HYDROCHLOROTHIAZIDE SUR L'INDUCTION DE L'APOPTOSE ET LA NEPHROTOXICITE PAR LA GENTAMICINE DANS LE CORTEX RENAL DE RATS

[Effect of Hydrochlorothiazide on Gentamicin-induced nephrotoxicity and apoptosis in renal cortex of rats]

ALLABI Aurel Constant [1], LALEYE Anatole [2], VAN BAMBECKE Françoise [3], MINGEOT-LECLERCQ Marie-Paule [3], TULKENS Paul [3]

1. Unité de Pharmacologie de la Faculté des Sciences de la Santé de Cotonou, Université d'Abomey-Calavi, BENIN.
2. Unité d'Histologie, d'Embryologie et de Cytogénétique de la Faculté des Sciences de la Santé de Cotonou, Université d'Abomey-Calavi, BENIN.
3. Unité de Pharmacologie cellulaire et moléculaire, Faculté de Médecine, Université Catholique de Louvain, Belgique.

Corresponding author contacts: Dr Aurel Constant ALLABI 01 BP 188 Cotonou Campus du champ de Foire-FSS Cotonou- BENIN
Email : acallabi@hotmail.com Tel: +229 95 73 49 00/ 90 08 35 09

RESUME

L'induction de l'apoptose au niveau du cortex rénal a été étudiée chez des rats Wistar de sexe masculin traités pendant 4 jours avec de la Gentamicine (GM) et/ou de l'Hydrochlorothiazide (HCTZ).
La GM a été administrée par voie intrapéritonéale à des doses (10 ou 20 mg/kg/jour pendant 4 jours) connues pour induire l'apoptose et des modifications fonctionnelles au niveau des reins.
L'HCTZ a été administré par gavage à des doses de 20 ou 40 mg/kg/jour pendant 4 jours.
La détection et la quantification de l'apoptose ont été faites en utilisant des techniques cytologiques (colorations Hématoxyline-éosine et Brachet) et immunohistochimiques (TUNEL).
L'induction de l'apoptose et la diminution de l'osmolarité urinaire sont significativement remarquables pour le traitement combiné GM+HCTZ comparé au traitement non combine à base de GM. Par contre, la GM et les phospholipides corticaux ne sont pas différents entre les deux types de traitement (GM+HCTZ vs GM).
Une tendance à l'augmentation de la créatinine sérique est observée durant le traitement GM+HCTZ comparée au traitement à base uniquement de GM ; ceci probablement comme le résultat d'une augmentation significative de la réponse apoptotique en cas de GM+HCTZ.
Nos résultats montrent que la toxicité rénale de la GM peut être notablement augmentée par une coadministration de l'HCTZ.
Mots clés : Gentamicine, Hydrochlorothiazide, Apoptose rénale, Néphrotoxicité des médicaments

ABSTRACT

Kidney cortex apoptosis was studied in male Wistar rats treated for 4 days with GM and/or hydrochlorothiazide.
Each rat was given GM intraperitoneally at doses that induce apoptosis and functional modifications in the kidneys (10 or 20 mg/kg/day for 4 days). Hydrochlorothiazide was given at a daily dosage of 20 or 40 mg/kg by gavage, for 4 days.
Apoptosis was detected and quantified using cytological (hematoxylin eosin and Brachet) and immunohistochemically (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling) staining, in parallel with a measurement of drug-induced phospholipidosis (cortical phospholipids), kidney dysfunction (serum creatinine and urinary osmolality) and GM accumulation in the renal cortex.
As compared to rats given GM alone, alteration induced combined hydrochlorothiazide and GM treatments were significantly different for the following criteria: apoptotic response, urinary osmolality while no significant differences were found for these criteria: renal cortical accumulation of GM and phospholipids.
A tendency of increased serum creatinine was observed when HCTZ was associated with GM than with GM alone, probably as a result of the significant increase of apoptosis during the combined treatment.
Our results show that the renal toxicity of GM can be noticeably increased by concomitant administration of hydrochlorothiazide
Key Words : Gentamicin, Hydrochlorothiazide, Apoptosis, Nephrotoxicity

INTRODUCTION

Aminoglycosides have long been an essential component of our armamentarium against severe, life-threatening infections caused by gram-negative bacilli. However, their clinical usefulness is tempered by the associated nephrotoxicity which occurs in 10-15% of patients undergoing treatment (Bennett, 1989). Aminoglycosides, and gentamicin (GM) in particular, are the drugs most frequently associated with adverse drug reactions (ADRs). The frequency of ADRs caused by GM is related to age, initial impairment of renal function and association with multiple-drug administration, hospital stay length and duration of therapy. Moreover, GM is frequently used in patients with severe clinical status, who are most at risk for side effects (Bertino JS et *al.*, 1993).

The major clinical manifestation of nephrotoxicity is a fall in glomerular filtration rate (GFR) and this usually brings patients to medical attention. The pathogenesis of aminoglycoside-induced nephrototoxicity is well documented (Tulkens, 1989). A disturbance of electrolyte homeostasis is a second, potentially serious but less well-documented side-effect of aminoglycoside therapy. A syndrome associating hypomagnesaemia, hypocalcaemia and hypokalaemia in patients on aminoglycoside therapy has been described (Kelnar et al., 1978; Watson A et al., 1983; Zaloga GP et al., 1984). A common finding in these studies is that the levels of urinary electrolyte excretion are inappropriately high for their low plasma concentration, indicating a failure of the kidneys to conserve cations adequately.

However, it is very difficult to establish a relationship between aminoglycoside treatment and renal electrolyte disturbances because of the concomitant use of other drugs and the presence of underlying disease processes. Animal studies have therefore proved useful. Early animal studies focused on the renal effects of chronic aminoglycoside administration on rats or dogs were performed. Hyperkaliuria (Brinker et al., 1981), hypercalciuria and hypermagnesiuria (Chahwala and Harpur, 1983; Harpur et al., 1985) have all been shown to accompany long term GM treatment.

Elliot et al.(1995, 2000) showed that GM caused isolated calciuria by an unknown mechanism. They also found that the calciuric effects of GM and furosemide were additive, whereas the noncalciuric diuretic chlorothiazide had no effect on GM calciuria. These previous work indicate that GM and diuretic impact on calcium homeostasis using different mechanisms. Recently Parsons et al (2000) concluded that acute GM-induced hypercalciuria is mediated by a decrease in calcium reabsorption in the early distal tubule. But they think that there is no relationship between GM-induced hypercalciuria and renal tubular injury.

The aims of the present study were to investigate the effect of hydrochlorothiazide administration on GM-induced apoptosis in renal proximal tubules and on acute renal failure in rats and secondly to evaluate the relationship between apoptosis and serum creatinine.

MATERIALS AND METHODS

Animals and treatments

All studies were performed with male Wistar rats (180 to 200g body weight) purchased from a commercial breeding farm (Iffa-Credo, l'Arbresle, France). Before and during treatment, animals were housed in a central facility submitted to a 12-h light-dark cycle, provided with regular rat chow and tap water ad libitium, and handled and treated according to the guidelines of the Belgian Ministry of Agriculture (agreement no. LA 12303116). Twenty-four hours before the end of the treatment, rats were transferred to metabolic cages designed for urine collection.

Before performing the definitive experiment, a pilot study was carried out. We applied five different doses of HCTZ (0.00 mg/kg body wt/24h = polyethylene glycol 300 1ml/200g body wt/24 h; 1mg/kg body wt/24h, 10 mg/kg body wt/24h; 20 mg/kg body wt/24h; 40 mg/kg body wt/24h), each given to three animals for 96 hours. The doses of 20 and 40 mg/kg body wt/24h had a statistically significant effect on diuresis, urinary osmolality, urinary excretion rates of Na and K and apoptosis compared to 0.00 , 1 and 10 mg/kg body wt/24h HCTZ treatment. Therefore, 20 and 40 mg/kg body wt/24h HCTZ were used in the definitive experiment of co-administration. For the definitive study, we selected two dosages of GM (10 and 20 mg/kg body wt/24h) based on previous experience (El Mouedden M, 2000).

For GM (Schering-Plough Labo, Heist-op-den-Berg, Belgium), the daily doses of 10 or 20 mg/kg were split into halves given as two separate administrations at around 9 a.m. and 5 P.M., respectively. GM was injected intraperitoneal by after appropriate dilution in 0.9% NaCl in order to deliver a volume about 0.5

ml per 200 g of animal and per injection. The actual volume injected into each rat was adjusted according to the body weight which was recorded immediately before each drug administration.

Hydrochlorothiazide (HCTZ; Sigma, St. Louis, MO, USA) 20 or 40 mg/kg was given by gastric gavages in a single administration at around 9 A.M. HCTZ was dissolved in polyethylene glycol 300 (Fluka Chemies Buchs) after appropriate dilution.

For control animals, 0.9% NaCl (0.5 ml per 200g of animal) and/or polyethylene glycol (1ml per 200g of animal) were administered. Each experimental group, including controls, contained six animals. Treated and control rats were killed approximately 16h after the last drug injection.

Sacrifice and sampling of renal tissue

Animals were killed by decapitation, and blood samples were collected from the stump for the measurement of serum creatinine. Both kidneys were exposed by laparotomy and excised. After longitudinal bisection, the renal cortex was removed by sharp dissection. For light microscopy studies, two necropsy specimens (each equivalent to approximately a quarter of each kidney) were fixed separately in Carnoy's mixture and in 10% neutral buffered formalin (4.2% formaldehyde) at room temperature for 24 and 48h, respectively. The remaining renal cortex tissue was snap-frozen in dry ice and stored at –80°C for future biochemical analysis.

Morphological studies. Histological demonstration of apoptotic cells

Kidney specimens, fixed in formalin or Carnoy's mixture, were dehydrated in graded ethanol solutions and embedded in paraffin. Sections (approximately 7µm thick) of formalin-fixed tissue were used for hematoxylin-eosine staining or terminal deoxynucleotidyl TdT-mediated dUTP-biotin nick end labelling (TUNEL), whereas other sections (also approximately 7µm thick) from tissue fixed in Carnoy's mixture were stained with methyl green-pyronine to the method of Brachet (Brachet J, 1953).

Immunohistochemical demonstration of apoptosis

Apoptotic cells were detected by TdT-mediated extension of 3'OH ends of fragmented DNA using either digoxigenin-or fluorescein-labeled dUTP as a precursor (TUNEL) (method adapted from the original work of Gavrieli et al., who used biotin-labeled dUTP). DNA-bound digoxigenin or fluorescein was revealed by reaction with antidigoxigenin or antifluorescein antibodies with peroxidase. Reagents were purchased as commercial kits (Apoptag; Oncor, Gaithersburg, Md.; in situ cell death detection kit POD; Boehringer Mannheim, Mannheim, Germany (presently Roche Diagnostics).

The immunohistochemical staining procedure was carried out in accordance with the suppliers' recommendations. Briefly, sections of formalin-fixed and paraffin-embedded specimens were dewaxed, rehydrated, and pre-treated with 20µg of protéinase K per ml for 15mn at room temperature. After rinsing, the sections were incubated for 2h in a reaction buffer containing TdT, dATP, and digoxigenin-11-dUTP or fluorescein-11-dUTP. At the end of the incubation, the sections were rinsed with a stop-wash buffer for 30 min at 37°C, and the mixture was replaced by peroxidase-conjugated antidigoxigenin or antifluorescein antibody.

Immunocomplexes were visualized by exposure to H_2O_2 and diaminobenzidine. Finally, sections were counterstained with methyl-green prior to mounting for light microscopy examination. Two positive controls were made to check that the immunostaining performed under our experimental conditions specifically revealed nuclei containing fragmented DNA. For the first one, DNA nicking was produced by exposing sections of control kidneys to micrococcal nuclease (10µg/ml, 10min, 37°C). In the second one, apoptosis was induced in cultured thymocytes by exposure to dexamethasone. In both cases, the appearance of a large number of immunostained nuclei was observed. Negative controls were also run by omitting the addition of TdT in the reaction mixture. No labelling was seen in this case in both kidney sections and smears of dexamethasone-treated thymocytes. However, in practice, this technique requires great expertise, as false-positive and false-negative results are common, and therefore simultaneous identification of the typical morphologic features is recommended.

Quantitative analyses

Enumeration of labelled nuclei (TUNEL histological) was performed for slides picked at random for each experimental animal (three slides for Brachet staining; two slides for apoptosis immunolabelling). These slides were assigned an arbitrary code which was disclosed to the observer (M.E.**) only after final pooling of

the results. Examination was made on a Zeiss light microscopy at 40 or s63 magnification for apoptotic nuclei. For calibration purposes, a square grid was mounted in on eyepiece, determining a square field of 0.04 or 0.016mm^2, depending on the magnification. The number of fields examined per slide was 20 for the methyl green-pyronine-stained sections and the immunostained sections.
For the histological assessment of apoptosis, we counted all cells with a pyknotic and karyorrheetic nucleus (typically, the total numbers of nuclei actually counted per animal were ~3 for controls and ~60 and 100 for rats treated for 4 days with 10 and 20 mg of GM per kg, respectively). For the assessment of apoptosis by TUNEL, we counted all nuclei exhibiting a frank brown labelling. These nuclei most often displayed typical alterations such as pyknosis, crescent-like condensation of the chromatin, or formation of apoptotic bodies.

Biochemical and microbiological studies
Cortical tissue samples were thawed and homogenized at 0°C in distilled water for measurement of their contents in total lipid phosphorus and protein, using well established procedures (Bartlett GR, 1959; Bligh EG, 1959; Lowry OH, 1951), which were fully validated in the previous study [variation coefficient of 1.5% and 1.8% for the assays of tissue phospholipids, and tissue proteins, respectively (n=20 in each case)].
The urine collected during the 24h period preceding the sacrifice was carefully mixed and stored at –20°C until analysis. Urine were thawed, mixed thoroughly and centrifuged at 25.000 rpm for 1h. The resulting pellets were resuspended in 1ml of distilled water and used for the determination of total lipid phosphorus, as detailed previously (Ibrahim S et al, 1991; Ibrahim S et al, 1989; Kishore BK et al, 1992). The coefficient of variation of this determination was 4.7% (n=20).
Urinary osmolality was measured by an automatic analyser. The variation of coefficient of this determination was 1%.
Serum creatinine was determined by the routine procedures (Bartels H et al, 1972; Digiorgio J, 1974; Jaffé M, 1886) used for human clinical samples in our University Hospital (Cliniques universitaires Saint-Luc, Brussels, Belgium), with intrarun and interrun variations of coefficients of 0.6 and 1.2 and 3 and 2.5%, respectively (Hitachi 717 autoanalyzer; Hitachi Ltd., Chiyoda-Ku, Tokyo, Japan).
GM content in renal cortex tissue was assayed by a plate diffusion bioassay, using Bacillus subtilis (ATCC 6633) as the test organism, using the technique developed earlier for cultured cell extracts (Tulkens P and Trouet A, 1978). All samples were assayed in triplicate against a series of known standards (extracts from control animals spiked with the corresponding antibiotic after homogenisation; no interference of homogenate protein was noted) over a 2.5-to 20-µg/ml range (typical r=0.987) and covering the range of drug concentrations found in the samples from treated animals.

Statistical analysis
The data are given as means ± SEM. Statistical differences between groups were evaluated by analysis of variance (ANOVA), using standard computer software. Differences were considered to be significant when $P< 0.05$.

RESULTS
The morphological appearance of the renal cortex of the animals treated at the different dosages of GM and /or HCTZ was studied by light microscopy.

The onset of apoptotic cell death in the kidney cortex of animals treated with GM and/or HCTZ was then quantified. Data are shown in ***Figure 1***.

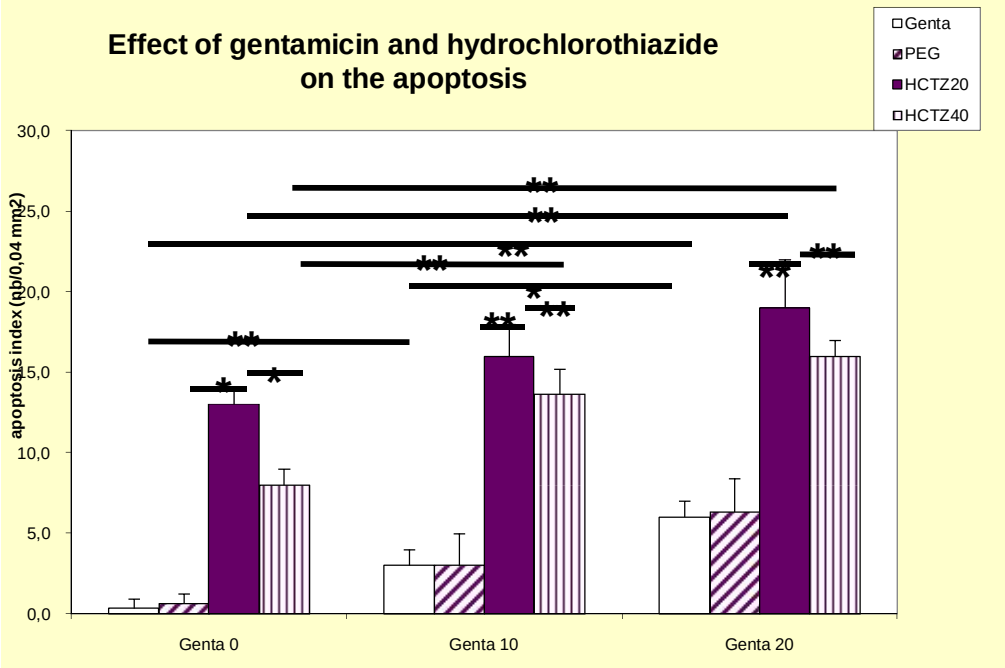


Figure 1: Enumeration of apoptotic nuclei observed in paraffin sections by the TUNEL method, in control animals (genta 0 and/or PEG) and in animals treated with gentamicin (10 or 20 mg/kg.day) and/or hydrochlorothiazide (20 or 40 mg/kg. day). Each histogram represents an animal group treatment and enumeration apoptotic variation standard. N=6 per group.

When the daily doses of GM and/or HCTZ were increased, apoptotic process became more frequent. But when the dose of HCTZ was raised to 40 mg/kg.day, we observed apoptotic process markdown and at, the same time the necrotic cells became more frequent and were found in several parts of the cortex (***Table 1***; ***Fig 1***). Concomitant administration of HCTZ at 20 mg/kg body wt/day and GM at 20 mg/kg body wt/day resulted in more apoptotic cells than were caused by other combination therapies (***Fig 1***).

Table 1: Morphology characteristics of renal cortex of rats treated by GM and/or HCTZ : Hematoxylin-eosin coloration

Treatement	***Rats G10***	***Rats PG10***	***Rats H20G10***	***Rats H40G10***	***Rats G20***
Necrotic cells [a]	--	--	+	+	+++
Suspected apoptosis cells [b]	--	--	+	++	+

+: means presence of necrotic or apoptosis cells
- : means absence of necrotic or apoptosis cells
G10 : Gentamicin at 10 mg/kg body wt/day
G20: Gentamicin at 20 mg/kg body wt/day
PG10: Polyethlene glycolle at 10 mg/kg body wt/day
H20: Hydrochlorothiazide at 20 mg/kg body wt/day
H40: Hydrochlorothiazide at 20 mg/kg body wt/day

Finally, the occurrence of the apoptotic process was additive in case of GM and HCTZ co-administration therapy. Apoptotic cells distribution in the renal cortex was clearly drug and dose dependant. HCTZ induced more apoptosis in distal convoluted tubule than in proximal tubule while GM nearly affected the

proximal tubule. Maximal apoptotic cells induce by GM were seen in proximal tubule or lumen. Distributions of apoptotic cells are shown in ***Figure 2***.

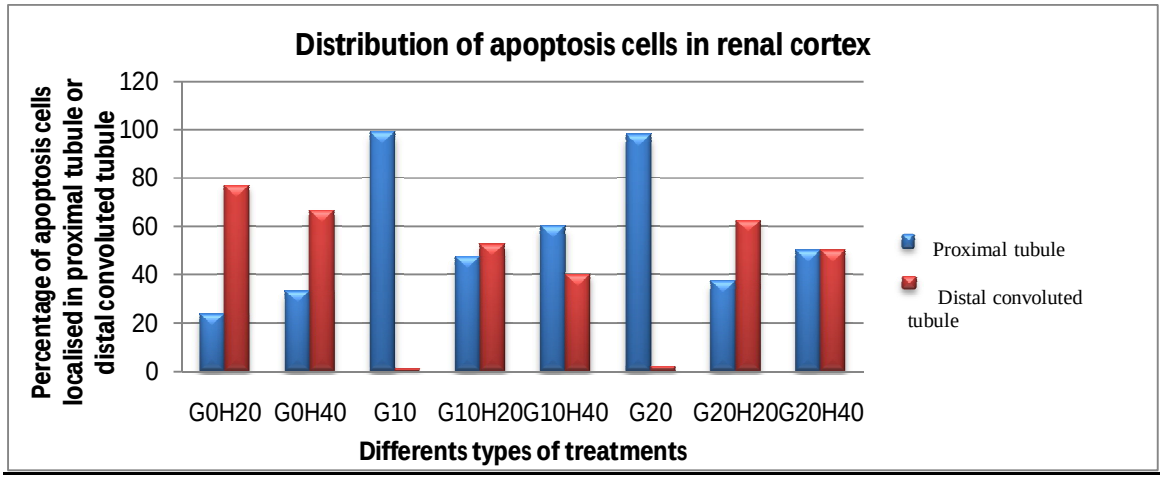


Figure 2: Distribution of apoptosis cells in renal cortex.

Four days of treatment with HCTZ and/or GM altered the structure of the tubular epithelium. Apoptosis cells within the epithelial lining were often seen. Occasionally, apoptotic cells and desquamated cells were seen within the tubular lumen. Mitotic cells were rarely seen. Sometimes, the tubular brush border was lost. In other respects, we observed the presence of many red blood cell casts in glomeruli.

Biochemical analysis

Results are shown in ***Figure 3.*** In our experiment, the level of serum creatinine increased after co-administration in a dose dependant manner. An increase of the doses of HCTZ and/or GM resulted in a rise of serum creatinine. Serum creatinine in the different groups was not significantly different. Nevertheless, we observed a global trend towards a potentialisation of renal toxicity when HCTZ was co-administered with GM.

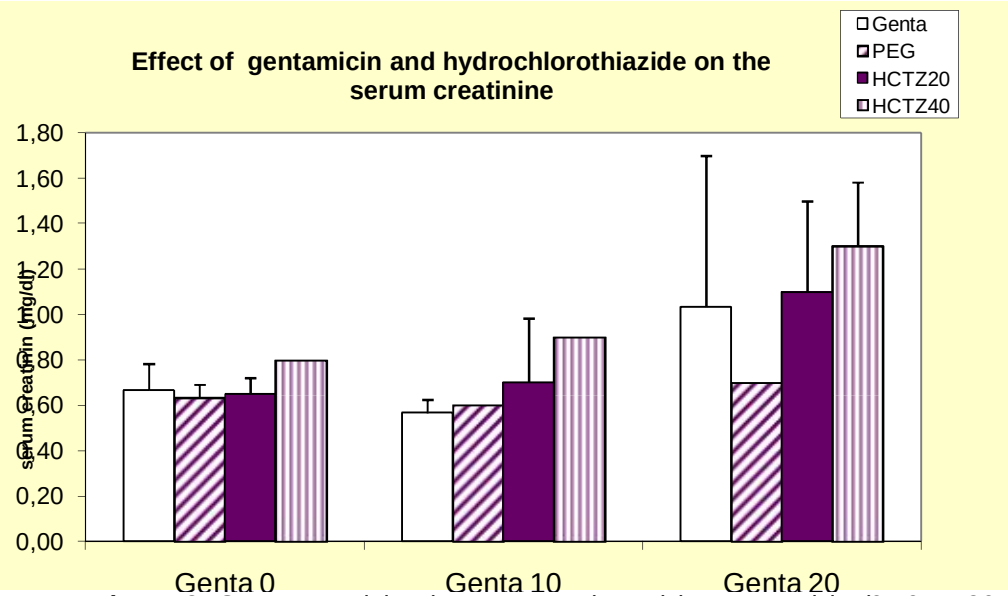


Figure 3: Serum creatinine in rats treated receiving gentamicin (0.10 or 20 mg/kg.day) and/or hydrochlorothiazide (0.20 or 40 mg/kg.day) for 4 days. Values are means ± SD (n=6 per group).

As previously described, the administration of GM caused the intralysosomal accumulation of phospholipids (phospholipidosis) which can be directly assessed by the measurement of total cortical phospholipids (Laurent et al, 1982). As shown in ***Figure 4***, the overall comparison did not show a significant difference of phospholipidosis related to treatment.

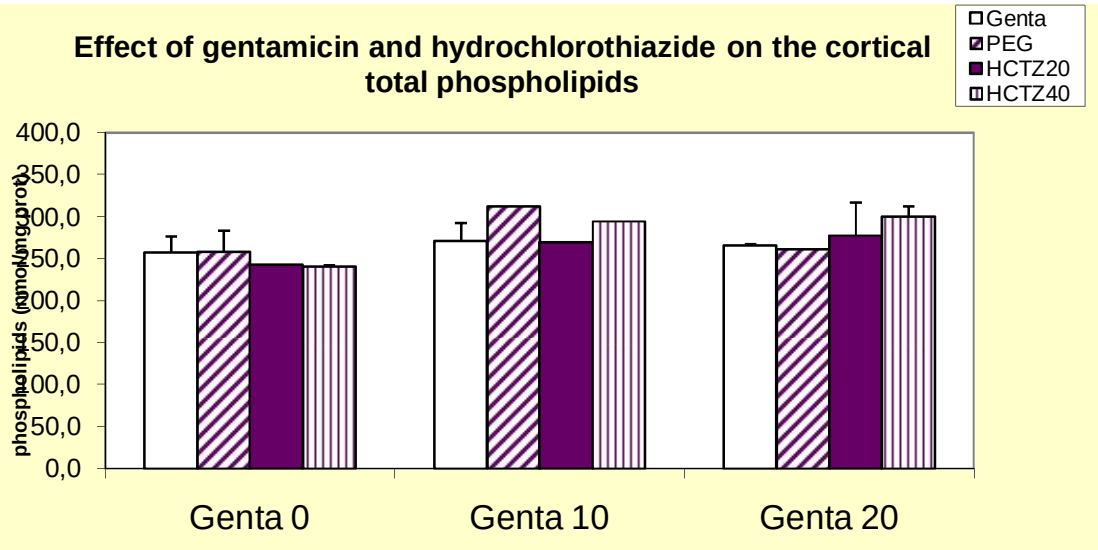


Figure 4: Cortical total phospholipids in rats treated receiving gentamicin (0.10 or 20 mg/kg. day) and/or hydrochlorothiazide (0.20 or 40 mg/kg.day) for 4 days. Values are means +/- SD (n=6 in each group).

Table 2: Cortical gentamicin content in rats treated

Treatment	Rats G10	Rats *PG10*	Rats *H20G10*	Rats *H40G10*	Rats *G20*	Rats *PG20*	Rats *H20G20*	Rats *H40G20*
Gentamicin Concentration In renal cortex (µg/mg)	**1,35±0,07**	**1,80±0,40**	**1,20±0,01**	**1,42±0,03**	**3,60±0,03**	**3,41±0,00**	**3,15±0,60**	**3,40±0,05**

The rats were treated with gentamicin (10 or 20 mg/kg.day) and/or hydrochlorothiazide (20 or 40 mg/kg.day) for 4 days. Values are means ± SD (n=6 in each group).

Examination of sections at high magnification revealed the presence of cells with typical alterations of apoptosis (cell shrinking eosinophilic cytoplasm and presence of a small and shrunken nucleus with chromatin condensation) (***Figure n°5***). The chromatin condensation characteristic of apoptosis is obvious in this cells as “pyknosis,” i.e., very dense staining of chromatin by hematoxylin. However, nuclei of quiescent cells (e.g., small lymphocytes) might be mistaked for pyknotic cells. Therefore, other histological techniques have been necessary to confirm apoptosis on equivalent slide.
The use of the periodic acid-Schiff reaction confirmed these cells localisation (data not shown).

Animals were treated with hydrochlorothiazide (20mg/kg.day) and GM (20mg/kg.day) for 4 days. Arrows point to cells showing clear evidence of nuclear alterations (chromatin condensation or pycnosis) related to apoptosis. There are many cells which are suggestive of apoptosis.

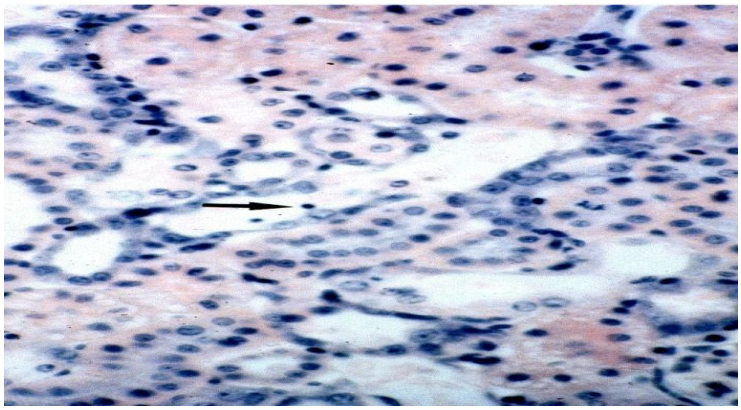


Figure 5: Light microscopy appearance of paraffin sections of kidney cortical specimens stained with hematoxylin eosin. Specimen kidney cortex of rats treated for 4 days with HCTZ (20 mg/kg.day) and GM (20 mg/kg.day): there are many cells which are suspected of apoptosis. Hematoxylin-eosin's staining (x 500)

The nuclear alterations characteristics of apoptosis were more clearly visible after using the Brachet technique (***Figure n°6***).

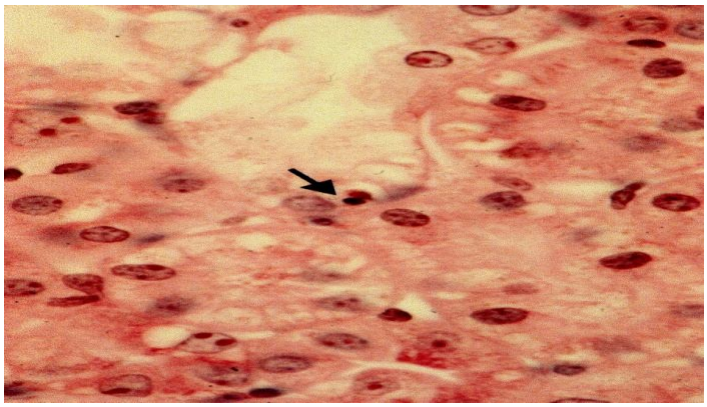
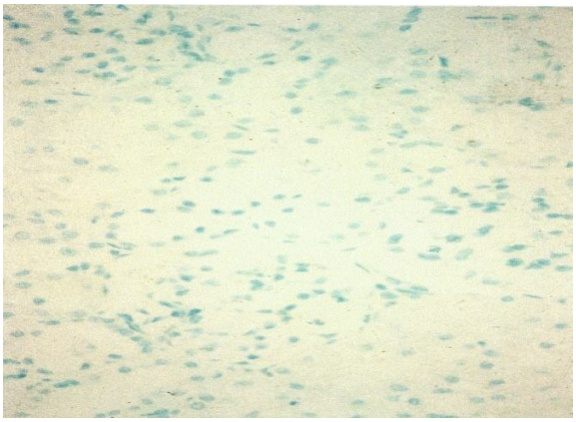


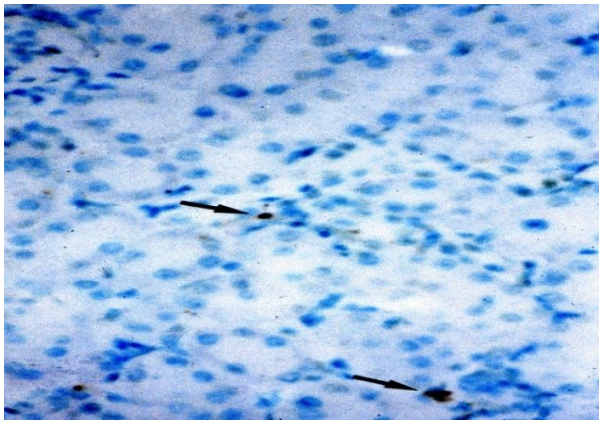
Figure 6: Light microscopy appearance of paraffin sections of kidney cortical specimens stained with methyl green-pyronin (Brachet's staining). Single arrow point to cell showing clear chromatin condensation suggestive to apoptosis. Animals were treated with GM (20 mg/kg.day) for 4 days.
Brachet's staining (x 1250)
Specimen kidney cortex of rats treated for 4 days with GM (20 mg/kg.day).

In parallel with these conventional staining techniques, we examined kidney sections after in situ immunohistochemical demonstration of DNA fragmentation (TUNEL technique) (***Figure n°7/A***; ***Figure n°7/B***).

Scattered peroxidase-labeled nuclei were easily recognized be detected over the entire cortex from all treated animals, due to their brownish appearance contrasting with the light background. In practice, TUNEL was used to confirm the nuclear alterations seen by the histological techniques.
Sections from control animals showed only a very few labeled nuclei, sometimes even no labeled nuclei (***Figure n°7/A***).



A: Negative TUNEL (x 125)
Specimen kidney cortex of rats treated with 0.9% NaCl and PEG 300.



B: Positive TUNEL (x 500)
Specimen kidney cortex of rats treated for 4 days with HCTZ (20 mg/kg.day) and GM (20 mg/kg.day).

Figure 7: Immunohistochemical demonstration of apoptotic nuclei in sections of kidney cortical specimens of controls (Animals treated with 0.9% NaCl and PEG 300) (***A***) and animals treated for 4 days with hydrochlorothiazide 20 mg/kg.day and GM 20 mg/kg.day (***B***), performed by TdT-mediated labeling of fragmented DNA (TUNEL). Positive nuclei were identified as shrunken, dark stained bodies with either a condensed or a fragmented appearance.

DISCUSSION

Mechanisms involved in cellular damage and apoptosis

After glomerular filtration, aminoglycosides are taken up at proximal tubule level by a process of adsorptive endocytosis binding the apical membranes by interacting with anionic phospholipids (Sastrasinh M *et al.*, 1982). Aminoglycosides inhibit the enzymatic breakdown of phosphatidylinositol. Miao *et al.*, have shown that inhibition of phosphatidylinositol-specific phospholipase C can per se cause apoptosis.

It is well-recognized that aminoglycosides cause an irreversible inhibition of lysosomal sphingomyelinase in cultured cells as well as *in vivo* (Laurent *et al.*, 1982). It can therefore be hypothesised that the resulting change in ceramide production from sphingomyelin could play a critical role in cell apoptosis occurence.

It has also been established that some cysteine proteases contained in lysosomes are capable of activating caspase-3 (Ishiaka R *et al.*, 1998), a key effector in the execution phase of apoptosis since it activates CAD (Enari M *et al.*, 1998). According to this scheme, aminoglycosides could thus trigger apoptosis by acting not at the commitment phase, but at the execution phase.

Free radicals derived from oxygen products during the treatment with aminoglycosides could be involved in the apoptotic process. These could explain why vitamin E and probucol, by their antioxidant activity have a protective effect against nephrotoxicity induced by GM (Abdel-Naim AB *et al.*, 1999).

About cortical phospholipids, the observations reported here differ from those by El Mouedden M *et al.*, 2000 who noticed a correlation between the degree of the lysosomal phospholipidosis and the occurrence of apoptosis in renal cortex. We think that 96 hours of treatments isn't sufficient for the development of conspicuous lysosomal alterations. Most probably HCTZ induced the subtoxic alteration of the renal cortex but this has yet to be proved, the induction of lysosomal phospholipidosis by HCTZ. However, we observe that GM induced lysosomal phospholipidosis. All these observations allow us to conclude that the occurrence of phospholipidosis is posterior to the apoptosis.

Angiotensin II promotes apoptosis in a variety of cultured cell types, including cardiomyocytes, endothelial cells, and fibroblasts (Solary E *et al.*, 1993; Thomas SE *et al.*, 1998). Both AT-1 and AT-2 receptors have been implicated in Angiotensin II-induced apoptosis (Kajstura J *et al.*, 1997; Yamada T *et al.*, 1996). Ortiz A *et al.*(2000), recently observed that therapy with either quinapril or the AT-1 receptor antagonist such as losartan reduced the rate of tubular

epithelial cell apoptosis in spontaneously hypertensive rats with renal mass reduction. Losartan also decreased apoptosis of tubular and interstitial cells that was associated with cyclosporine A nephrotoxicity in rats (Thomas SE *et al*., 1998). In fact, high dose of HCTZ provokes a relatively high loss of sodium and as a result a decrease of blood volume. In response, we observed aldosteronism up regulation and angiotensin II increase. By this mechanism, HCTZ can induce apoptosis.
However, another mechanism can explain the induction of apoptosis by HCTZ. Loffing J *et al*. (1998) have shown that thiazide diuretics provoked similar lesions in the distal convoluted tubule (DCT). They favour the explanation that the primary cause for the injury of DCT cells during treatment with thiazide diuretics might be related with their pharmacological effect, that is, inhibition of apical NaCl entry and increase of apical Ca^{2+} entry.

In our study, we observed that HCTZ induces more apoptosis in DCT than proximal tubule, while HCTZ are secreted by proximal tubule cells (Velazquez H, 1995). Most importantly, apoptosis induction in the renal cortex by HCTZ is dependant on HCTZ applied doses. These data confirm that apoptosis induced by HCTZ is due to pharmacological effects.
Strange (1989) showed that inhibition by amiloride of apical Na entry in isolated rabit CCD cells is followed by immediate cell shrinkage. Cell shrinkage seems to induce catabolic processes that ultimately might lead to apoptotic cell death.
In the present study, we used HCTZ in order to block the apical NaCl entry into the renal cortex cells in rats with possible renal injury (GM treatment). Our experiments can be compared with through Finn WF and Luc J (1995) study: they showed that dietary sodium restriction promotes apoptosis after renal ischemia, and on the contrary, high Na^+ intake reduces the incidence of apoptosis after ischemia.
Duc C *et al*. (1994) have shown the presence of EnaC (amiloride-sensitive sodium channels) in the distal segments of renal cortex. This receptor can assure Na entry into the cells during a blockade of the rTSC1-mediated transports. Moreover, it is possible that a different Na^+ transport protein increases by on up regulation process after HCTZ administration. These data argue against an implication of the Na^+ entry mechanism in the introduction of apoptosis.
HCTZ have a hypocalciuric effect which is thought to be promoted by increasing Ca^{2+} entry through the apical membrane of distal cells (Lajeunesse D and Brunette MG, 1991; Gesek FA and Friedman PA, 1992). It can be speculated that the structural degradation and apoptotic cell death of distal convoluted tubule cells might not solely be caused by the reduction of apical $NaCl^-$ entry into the cells, but also (or alternatively) by an abrupt and transient rise in intracellular Ca^{2+} associated with HCTZ treatment. Data derived from cell culture suggest that an increase in free intracellular Ca^{2+} might trigger apoptosis and that inhibition of Ca^{2+} entry into cells might prevent apoptosis (Tsukidate K *et al.*, 1993; Nicotera P *et al.*, 1994).
Under our experimental conditions, probably, the hypercalcemia effect of HCTZ alters the acute hypercalciuric response to GM. But HCTZ doesn't interfere with GM accumulation in the renal cortex (Table 2) in contrast to furosemide (Nakahama H et al, 1989_b). This may suggest that the acute effects of GM on renal calcium handling do not contribute to the subsequent nephrotoxicity and GM-induced apoptosis.

Recently, mitochondrial permeability transition has been linked to the induction of apoptosis. It may be possible that GM induced apoptosis via this mechanism. Rustenbeck I *et al.*(1998) have demonstrated that ability of GM to inhibit Ca^{2+}mitochonfrial accumulation may contribute to the mitochondrial lesions which are known to occur early in the course of aminoglycoside-induced nephrotoxicity. It is important to investigate the relationship between GM effect on mitochondrial function (energetic, ion transport) and GM-induced apoptosis. It is possible that GM interferes with HCTZ on mitochondrial function.

Apoptosis: beneficial or deleterious role in renal injury?
The role of apoptosis in renal injury and its role in specific acute renal failure were not obvious. The presence of apoptotic cells have been described in many renal diseases: diabetic nephropathy, proteinuria, HIV nephropathy, proliferative glomerulonephritis (Ortiz A *et al.*, 2000).

It is clear that apoptosis maintains cellular homeostasis. Most tissues depend on well-ordered apoptosis and cell replacement for normal functioning. Indeed, approximately 100, 000 cells are produced every second in a human, and a similar number die through apoptosis. One advantage of apoptosis for the tissue is the absence of inflammation generation.

Evidence from experimental models of renal injury suggests that apoptosis co-exists with renal cell proliferation (El Mouedden M *et al.*, 2000). In this case, apoptosis represents a physiologic process to eliminate redundant cells and resolve an exaggerated proliferative response to injury. In this setting, apoptosis can restore cell number to pre-injury levels, but also can contribute to delayed recovery from acute renal failure. This might be especially true in the clinical setting. Contrary to the experimental situation, in which a single insult causes enough damage for the model to be reproducible, the clinical setting is often characterized by repeated low-grade renal damage leading to tubular epithelial cell apoptosis, even in the absence of clinical deterioration of renal function (Jaffe R *et al.*, 1997) and can hamper recovery in previously damaged tubules.

An excess rate of apoptosis of tubular epithelial cells has been observed in experimental models of chronic tubular atrophy and human HIV nephropathy (Vaux DL and Korsmeyer SJ, 1999; Schelling JR *et al.*, 1998; Thomas GL *et al.*, 1998). These cases can lead to think that apoptosis has a deleterious role in some renal diseases. Indeed, the precise relative contribution of both mechanisms (apoptosis and necrosis) to the renal injury is uncertain and might depend on the severity of the insult.

In the present study, apoptosis occurs before necrosis. When the insult increases, necrosis becomes more important than apoptosis (Table 1).

Kidney dysfunction

Experiments carried out in our laboratories have shown that GM given at high doses results in polyuria and of a minor decrease of the urinary osmolality. This effect is potentialised in case of co-administration of two drugs. If the mechanism by which HCTZ induces a reduction of the urinary osmolality is well known, the GM mechanism is less well known. If there is a relationship between urinary hypoosmolality induced by aminoglycosides and their toxicity, it seems logical to think of a potentialisation of nephrotoxicity in the case of administration of the two drugs. Medical supervision of urinary osmolality in patients taking a multiple drugs would then become important in order to prevent renal toxicity.

Globally, the level of serum creatinine increases after co-administration and it is dose dependent. When the doses of HCTZ and/or GM were increased, the level of serum creatinine seemed to increase as a result. However, serum creatinine levels of the different groups are not statistically different. Nevertheless, we can conclude in a global tendency of potentialisation of renal toxicity when coadministation of HCTZ and GM is done. In the present study, we observed that HCTZ can't modify GM accumulation in renal tissues (see Table 2) but potentiate GM nephrotoxicity.

Then HCTZ potentiate GM nephrotoxicity by another mechanism. Aminoglycoside nephrotoxicity could be aggravated by diuretics due to a fall in plasmatic volume (Nakahama *et al.*, 1989). Thiazides, when administered acutely, reduce glomerular filtration, mostly by a direct vascular effect and a rise in the intraluminal hydrostatic pressure. This could explain the potentiation of renal toxicity of the drug in the case of acute administration. It is very probable that this effect considerably is reduced in the case of chronic administration due to the diminution of pharmacological effects of thiazides.

We clearly observe that co-administration greatly improve the induced apoptosis. The incidence of apoptosis obtained in combination therapy is additive. It appears that apoptosis is an earlier and more sensitive phenomenom then rise in serum creatinine. Can apoptosis be considered as a for-runner of nephrotoxicity?

REFERENCES

1- Abdel-Naim AB, Abdel-Wahab MH, Attia FF. Protective effects of vitamin E and probucol against GM-induced nephrotoxicity in rats. Pharmacol Res 1999; 40:183-187.
2- Bartels H, Böhmer M, Heierli C. Serum kreatininbestimmung ohne Enteiweissen. Clin Chim Acta 1972; 37:193-197.
3- Bartlett GR. Phosphorus assay in column chromatography. J Biol Chem 1959; 234:466-468.
4- Bennett WM. Mechanisms of aminoglycoside-induced nephrotoxicity. Clin Exp Pharmacol Physiol 1989; 16:1-6.
5- Bertino JS, Booker LA, Franck PA, Jenkins PL, Franck KR, Natziger AN. Incidence of and significant risk factors for aminoglycosides-associated nephrotoxicity in patients dosed by using individualised pharmacokinetic monitoring. J Infect Dis 1993; 167:173-179.
6- Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37:911-917.
7- Bodi I, Abraham AA. Kimmel PL. Apoptosis in human immunodeficiency virus-associated nephropathy. Am J Kidney Dis 1995; 26:286-291.

8- Brachet J. The use of basic dyes and ribonuclease for the cytochemical detection of ribonucleie acid. Q J Microsc Sci 1953; 94:1-10.
9- Brinker KR, Bulger RE, Dobyan DC, Stacey TR, Southern PM, Henrich WL, Cronin RE. Effect of potassium depletion on gentamicin nephrotoxicity. J Lab Clin Med 1981; 98:292-301.
10- Chahwala SB, Harpur ES. Gentamicin induced hypercalciuria in the rat. Acta Pharmacol Toxico 1983; 53:358-362.
11- Digiorgio J. Nonprotein nitrogenous constituents, p. 503-564. In Henry RJ, Cannon DC, Winkelman JW (ed), Clinical chemistry: principles and techniques, 2nd ed. Harper and Row Publishers, inc., New York, N.Y. 1974
12- Duc C, Farman N, Canessa CM, Bonvalet J-P, Rossier BC. Cell-specific expression of epithelial sodium channel, and subunits in aldosterone-responsive epithelia from the rat: localisation by in situ hybridization and immunocytochemistry. J Cell Biol 1994; 127:907-921.
13- El Mouedden M, Laurent G, Mingeot-Leclercq MP, Taper HS, Cumps J, Tulkens P. Apoptosis in renal proximal tubules of rats treated with low doses of aminoglycosides. Antimicrob Agents Chemother 2000; 44:665-675.
14- El Mouedden M, Laurent G, Mingeot-Leclerck MP, Tulkens P. GM-induced apoptosis in renal cell lines and Embryonic Rat Fibroblasts. Tox Sciences 2000; 56:229-239.
15- Elliott WC, Patchin DS. Effects and interactions of GM, polyaspartic acid and diuretics on urine calcium concentration. J Pharm Exper Ther 1995; 273:280-284.
16- Elliott C, Newman N, Madan A. GM effects on urinary electrolyte excretion in healthy subjects. Clin Pharmacol Ther 2000; 67:16-21.
17- Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A Caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD. Nature 1998; 391:43-50.
18- Finn WF, Luc J. Sodium restriction promotes apoptosis after renal ischemia in the rat. (abstract) J Am Soc Nephrol 1995; 6:978.
19- Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 1992; 119:493-501.
20- Gesek FA, Friedman PA. Mechanism of calcium transport stimulated by chlorothiazide in mouse distal convoluted tubule cells. J Clin Invest 1992; 90:429-438.
21- Harpur ES, Fathalla SAK, Phipps DJ. Increased urinary excretion of calcium and magnesium is an early manifestation of gentamicin-induced nephrotoxicity in the rat. In Renal Heterogeneity and Target Cell Toxicity. ed. Bach, P.H. pp. 1985; 349-352. Chichester: John Wiley & Sons.
22- Ibrahim S, Clerckx-Braun F, Jacqmin P, Donnez J, Brulein V, Tulkens PM. Effect of the schedule of administration of two aminoglycosides, netilmicin and amikacin on urinary phospholipid excretion in humans, p. 93-97. In Bach PH, Gregg NJ, Wilks MF, Delacruz L (ed), Nephrptoxicity: mechanisms, early diagnosis and therapeutic management. Marcel Dekker, New York, N.Y. 1991.
23- Ibrahim S, Van der Auwera P, Meunier F, Tulkens PM. Effect of netilmicin and amikacin on urinary phospholipid excretion in humans. Arch Toxicol 1989; 13(Suppl.): 413-416.
24- Ishiaka R, Utsumi T, Yabuki M, Kanno T, Furuno T, Inoue M, Utsumi K. Activation of caspasc-3-like protease by digitonin-treated lysosomes. FEBS Lett. 1998; 435:233-236.
25- Jaffé M. Über den Niederschlag welchen Pikrinsäure in normalen Harn erzeugt und über eine neue Reaktion des Kreatinins. Z Physiol Chem 1886; 10:391-400.
26- Jaffe R, Ariel I, Berri R, Paltiel O, Hiss Y, Rosen S, Brezis M. Frequent apoptosis in human kidneys after acute renal hypoperfusion. Exp Nephrol 1997; 5:399-403.
27- Kajstura J, Gigola E, Malhotra A, Li P, Cheng W, Meggs LG, Anversa P. Angiotensin II induces apoptosis of adult ventricular myocites in vitro. J Moll Cell Cardiol 1997; 29: 859-870.
28-Kelnar CJH, Taor WS, Reynolds DJ, Smith DR, Slavin BM, Brook CGD. Hypomagnesaemic hypocalcaemia with hypokalaemia caused by treatment with high dose gentamicin. Arch Dis Child 1978; 53:817-820.
29-Kishore BK, Ibrahim S, Lambricht P, Laurent G, Maldague P, Tulkens PM. Comparative assessment of poly-L-aspartic and poly-L-glutamic acids against GM- induced renal lysosomal phospholipidosis, phospholipiduria and cell proliferation in rats. J Pharmacol Exp Ther 1992; 262 : 424-432.
30- Lajeunesse D, Brunette MG. The hypocalciuric effect of thiazides: subcellular localization of the action. Pflugers Arch 1991; 417:454-462.
31-Laurent G, Carlier MB, Rollman B, Van Hoof F, Tulkens P. Mechanism of aminoglycoside-induced lysosomal phospholipidosis: in vitro and in vivo studies with GM and amikacin. Biochem Pharmacol 1982; 31:861-870.
32- Laurent G, Maldague P, Carlier MB, Tulkens P. Increased renal DNA synthesis in vivo after administration of low doses of gentamicin to rats. Antimicrob Agents Chemother 1983; 24:586-593.
33- Laurent G, Kishore BK, Tulkens PM. Aminoglycoside-induced phospholipidosis and nephrotoxicity. Biochem Pharmacol 1990; 40:383-392.
34- Loffing J, Loffing-Cueni D, Hegyi I, Kaplan MR, Herbert SC, Le Hir M, Kaissling B. Thiazide treatment of rats provokes apoptosis in distal tubule cells. Kidney Int 1997; 50: 180-190.
35- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193:265-275.
36- Luft FC, Kleit SA. Renal parenchymal accumulation of aminoglycoside antibiotics in rats. J Infect Dis 1974; 130:656-659.

37- Miao JY, Kaji K, Hayashi H, Araki S. Inhibitors of phospholipase promote apoptosis of human endothelial cells. J Biochem 1997; 121: 612-618.
38-Mingeot-Leclercq MP, Tulkens PM. Aminoglycosides: nephrotoxicity. Antimicrob Agents Chemother 1999; 43:003-012.
39- Nakahama H, Shin S, Moriyama T, Kakihara M, Fukuhara Y, Orita Y, Kamada T. Enhancement of GM-induced inhibition of phosphatidylinositol hydrolysis in rabbit renal proximal tubular brush-border membrane by furosemide. Ren-Fail 1989; 11:105-109.
40- Nakahama H, Fukuhara Y, Orita Y, Yamauchi A, Takama T, Kamada T. Furosemide accelerates GM accumulation in cultured renal cells (LLC-PK1cells). Nephron 1989; 53:138-141.
41- Nicotera P, Zhivotovsky B, Orrenius S. Nuclear calcium transport and the role of calcium in apoptosis. Cell Calcium 1994; 16:279-288.
42- Ortiz A, Lorz C, Catalán MP, Danoff TM, Yamasaki Y, Egido J, Neilson EG.
Apoptotic regulatory proteins in renal injury. Nephrology forum. Kidney Int 2000; 58:467-485.
43- Parsons PP, Garland HO, Harpur ES, Old S. Acute GM-induced hypercalciuria and hypermagnesuria in the rat: dose-response relationship and role of renal tubular injury. Br J Pharmacol 1997; 122:570-576.
44- Parsons PP, Garland HO, Harpur ES. Localization of the nephron site of GM-induced hypercalciuria in the rat: a micropuncture study. Br J Pharmacol 2000; 130:441-449.
45- Rustenbeck I, Loptien D, Fricke K, Lenzen S, Reiter H. Polyamine modulation of mitochondrial calcium transport. II. Inhibition of mitochondrial permeability transition by aliphatic polyamines but not by aminoglucosides. Biochem-Pharmacol 1998; 56:977-995.
46- Sastrasinh M, Weinberg JM, Humes HD. The effect of gentamicin on calcium uptake by renal mitochondria. Life Sci. 1982; 30:309-315.
47- Schelling JR, Nkemere N, Kopp JB, Cleveland RP. Fas dependent fratricidal apoptosis is a mechanism of tubular epithelial cell deletion in chronic renal failure. Lab Invest 1998; 78: 813-824.
48- Solary E, Bertrand R, Kohn KW, Pommier Y. Differential induction of apoptosis in indifferential and differential HL-60 cells by DNA topoisomerase I and II inhibitors. Blood 1993; 81:359-368.
49- Strange K. Ouabain-induced cell swelling in rabbbit cortical collection tubule. NaCl transport by principal cells. J Membr Biol 1989; 107:249-261.
50- Thomas GL, Yang B, Wagner BE, Savill J, El Nahas AM. Cellular apoptosis and proliferation in experimental renal fibrosis. Nephrol Dial Transplant 1998; 13:216-226.
51- Thomas SE, Andoh TF, Pichler RH, Shankland SJ, Couser WG, Bennett WM, Johnson RJ. Accelerated apoptosis characterizes cyclosporine-associated interstitial fibrosis. Kidney-International 1998; 53:897-908.
52- Tsukidate K, Ymamoto K, Snyder JW, Farber JL. Microtubule antagonists activate programmed cell death (apoptosis) in cultured rat hepatocytes. Am J Pathol 1993; 143:918-925.
53- Tulkens P, Trouet A. The uptake and intracellular accumulation of aminoglycosides antibiotics in lysosomes of cultured rat fibroblasts. Biochem Pharmacol 1978; 27:415-424.
54- Tulkens PM. Nephrotoxicity of aminoglycoside antibiotics. Toxicol. Lett. 1989; 46:107-123.
55- Velazquez H, Knauf H, Mutschler E. Thiazide diuretics, in Diuretics, edited by Greger RF, Knauf H, Mutschler E, Berlin, Springer-Verlag, 1995; p275.
56- Vaux DL, Korsmeyer SJ. Cell death in development. Cell 1999; 96:245-249.
57- Watson A, Holohan PD, Ross CR. Severe hypomagnesaemia and hypocalcaemia following gentamicin therapy. Ir Med J 1983; 76: 381-383.
58- Yamada T, Horiuchi M, Dzau VJ. Angiotensin II type 2 receptor mediates programmed cell death. Proc Natl Acad Sci USA 1996; 93:156-160.
59- Zaloga GP, Chernow B, Pock A, Wood B, Zaritsky A. Hypomagnesaemia is a common complication of aminoglycoside therapy. Surg. Gynaecol Obstet 1984; 158:561-565.