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Effect of Nephrotoxicants on α-Methylglucose Uptake in LLC-PK$_1$

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LLC-PK$_1$을 이용한 신독성 물질들의 α-methyl glucose uptake에 미치는 영향의 평가

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ABSTRACT

Many nephrotoxic agents exert their effect primarily on the cells of the proximal tubules. We used the LLC-PK$_1$, kidney epithelial cell line as a model system for studies on nephrotoxicity and investigated whether the uptake of α-methylglucose (α-MG) could serve as a parameter to assess effects of nephrotoxicants on the functional integrity of the cells at an early time of toxicity. The enzyme leakage test which has been used to be as a conventional cytotoxic parameter *in vitro*, was conducted to compare with α-MG uptake. Treatment with cisplatin for 24 and 48 hours significantly increased activities of lactate dehydrogenase and γ-glutamyltransferase in culture medium at a concentration of 50μM. However, above 100μM of concentration, activities of these enzymes in media were dramatically decreased by cisplatin. These observations indicate that cisplatin has direct inhibitory effect on the activities of these enzymes and make it doutful to use enzyme leakage test to demonstrate damage of kidney cells by chemicals such as cisplatin over the appropriate range of concentration. Cisplatin inhibited α-MG uptake at a low concentration which enzymes were not leaked. Also cadmium chloride and mercuric chloride which are acutely nephrotoxic *in vivo*, significantly inhibited α-MG uptake at a low concentration. These results indicate that the uptake of α-methylglucose in LLC-PK$_1$ cell line is a useful biomarker for the study of nephrotoxicity.

INTRODUCTION

The kidney is a highly differentiated organ with many different cell types of marked functional, morphological, and biochemical heterogenecity. Many nephrotoxic agents exert their noxious effect in a well-defined region of the kidney. This is due to the susceptibility of specific targets in certain cells to a nephrotoxin, their capacity to actively transport and concentrate a nephrotoxin, or their capacity to metabolize it into a (more) toxic compound. The cells of the proximal convoluted tubules are the first that are exposed to the glomerular ultrafiltrate in the tubular lumen. Because they actively transport many organic and inorganic compounds, and are able to perform a wide range of drug metabolizing reactions, they are a primary target of many nephrotoxic agents.

Recently, the use of cell culture techniques has permitted the study of a relatively homogeneous population of renal epithelial cells under carefully controlled environmental conditions.[1] The fixed polarity of cells of the LLC-PK_1 pig kidney epithelial cell line and their ability to form tight juntions enable a confluent monolayer of these cells to behave as an epithelial cell sheet and engage in unidirectional transport of salt and water, resulting in dome formation.[2,3,4] The LLC-PK_1 cells are also known to possess a Na^+-dependent active sugar transport system similar to that found in proximal tubules.[5,6,7,8] The cells contain relatively high levels of proximal tubule marker enzymes.[9] The properties of this cell line thus make it useful for the analysis of some renal epithelial functions.

A well-defined parameter should be used to assess functional integrity of the cells. However, both vital dye exclusion tests and enzyme leakage determinations are parameters that assess the very last stage of toxicity. Therefore, the purpose of this study was to define a parameter which is indicative of cellular damage at an early time point.

An important function of the proximal tubular cells is active transport of compounds from the primary urine to the blood and vice versa. Carriers for uptake at one side of the cell and carriers for excretion at the other side of the cell effect this vectorial transport. Therefore, in studies with intact renal tissue the capacity for transport of organic ions like *p*-aminohippurate (PAH), tetraethylammonium, and *N*-methylnicotinamide is often used to assess functional integrity of the biological meterial. However, these ions are not suited to characterize transport *in vitro* systems, because they are taken up and excreted by the cell at the same time from and into the incubation medium. Glucose, too, is normally transported both into the cell (at the luminal side) and out again (at the basolateral side). As a consequence of glucose itself is not suitable for determination of transport in single cells. However, α-methylglucose (α-MG, 1-*O*-methyl-α-D-glucopyranoside), a glucose analog that cannot be metabolized, is a substrate for the active sodium-glucose cotransport carrier at the apical membrane but not for the carrier for facilitated transport out of the cell at the basolateral membrane.[10,11] Therefore, α-MG is trapped in the cell.

In vivo, the appearance of glucose in the urine is one of the early signs of proximal tubular dysfunction. Therefore, we chose glucose transport as a parameter by which damage to prox-

imal tubular cells may be assessed sensitively and easily. In order to investigate whether α-methylglucose uptake by the cells was sensitive index of cytotoxicity at an early time point, we compared enzyme leakage determination following treatment with α-MG uptake.

In these studies, three potent nephrotoxicants, mercuric chloride, cadmium chloride and cisplatin have been used. Mercuric chloride and cadmium chloride are known to cause *in vivo* proximal tubular necrosis with marked glucosuria and proteinuria via different mechanisms.[12,13] Cisplatin, one of the most widely used antineoplastic agents, is reported to exhibit severe nephrotoxicity with its main target in the proximal tubules.[14,15]

MATERIALS AND METHODS

1. Chemicals

Cisplatin, mercuric chloride and cadmium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). α-Methylglucose was obtained from Amersham International PLC. (Amersham, U.K.). Lactate dehydrogenase assay kit and γ-Glutamyltransferase assay kit were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2. Cell culture

LLC-PK$_1$ cells at passage 195 obtained from the American Type Culture Collection (ATCC CRL-1392) were maintained by serial passages in plastic culture dishes (Corning Co.).[2] Monolayer cultures were grown in an atmosphere of 5% CO_2-95% air at 37℃. The complete medium consisted of medium 199 (Sigma Co.) supplemented with 3% fetal calf serum (Sigma Co.) with antibiotics. The cells were subcultured every 3 to 4 days using 0.025% trypsin in phosphate-buffered saline. The cells fed fresh complete medium on the 2nd day after inoculation, were exposed to fresh medium with or without chemicals on the 4th day and used for α-methylglucose uptake and enzyme assay. The cells reached confluency on the 4th day after subculture.

3. Enzyme assay

The activities of lactate dehydrogenase and γ-glutamyltransferase released into the culture medium were measured. The activity of lactate dehydrogenase (E.C. 1.1.1.27.) was determined spectrophotometrically at 340nm by measuring the conversion of pyruvate to lactate using Sigma diagnostic kit. γ-Glutamyltransferase (E. C. 2.3.2.2.) was estimated using γ-glutamyl-3 -carboxy-4-nitroanilide as substrate and glycylglycine as acceptor. The culture medium, in which LLC-PK$_1$ cells were incubated for various time with chemicals, was centrifuged at 3000rpm for 10min and then supernatant was used for enzyme assay.

4. Measurement of α-methylglucose uptake

After treatment, the monolayers were washed twice with Hanks'-HEPES buffer (pH 7.40) and subsequently incubated with Krebs-Henseleit bicarbonate buffer (pH 7.40) supplemented with 2.5% (w/v) BSA and 0.1mM[^{14}C] α-methylglucose. At the end of the uptake period, the monolayers were washed three times with ice cold Krebs-Henseleit-bicarbonate buffer (4℃) and subsequently incubated for 2hr at 37℃ with 1ml of 2N NaOH in order to solubilize the cells. The wells were washed twice with 100μl of 2N NaOH, and the washing pooled with the solubilized fraction. The radioactivity was determined

by liquid scintillation counting using INSTA -GEL XF (Packard, Downers Grove, Illinois, U. S.A.).

5. Protein assay

Protein concentration was measured by the method of Bradford (1976), using the Bio-Rad Protein Assay Kit, with bovine γ-globulin as a standard.

6. Statistics

All data represent a minimum of three seperate experiments with triplicate determinations and are expressed as means±SD of one typical experiment as indicated in the figure legends. The statistical significance between mean values of treated and control groups was evaluated by analysis of variance and Duncan's multiful range test with significance set at $p<0.05$.

RESULTS AND DISCUSSION

1. Effect of Cisplatin on Enzyme Release

It is of paramount importance for all *in vitro* systems to have well defined, relevant and reliable parameters to assess the various kinds of cellular damage due to a toxic insult. The most popular method to obtain information on cellular integrity is to determine whether the plasma membrane is intact or not. An intact, viable cell is impermeable to most large and/or charged molecules. When the structure of the cell membrane is affected, the cell can become leaky. As a result large cytosolic molecules like enzymes can leak out. For enzyme leakage determinations lactate dehydrogenase (LDH) is most often used. The alternative situation, the exclusion tests with vital dyes, like trypan blue, erythrosin B or nigrosine, are also easy to perform and allow a rapid estimation of the percentage of viable cells. The LDH assay is more convenient if tubular fragments are used than the vital dyes, because the individual cells in the fragments are difficult to distinguish. In this study assay of LDH activity in culture medium was conducted to estimate the toxicity of cisplatin in the LLC -PK_1 cells. γ-Glutamyltransferase (γ-GT) was also measured in culture medium, because γ-GT is one of the marker enzyme for proximal tubular cells.[16]

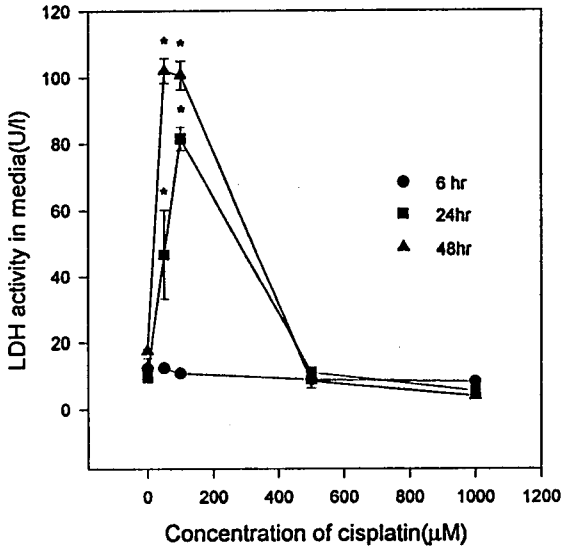

Fig. 1. Lactate dehydrogenase (LDH) release in LLC -PK_1 cells treated with different concentrations of cisplatin for 6, 24, 48 hours. Values are expressed by means±SD of three seperate experiments. *Significantly different from the control at $p<0.05$.

As shown in Fig. 1, upto 100μM of concentration, cisplatin produced dose-dependent increases in activity of LDH in culture medium for 24 hours treatment. The activity of LDH in culture medium was increased to 460% and 810% of control by treatment with 50μM and 100

μM of cisplatin, respectively. However, above 100μM concentration of cisplatin, the activity of LDH in medium was markedly decreased to control level, despite of increase of cytotoxicity. Treatment with 50μM and 100μM of cisplatin for 48 hours significantly enhanced (5-fold) LDH activity in media. But, treatment with 500μM of cisplatin for 48 hours dramatically reduced LDH activity in media to 50% of control. No change of LDH activity was caused by cisplatin treatment for 6 hours upto 1mM concentration.

The activity of γ-GT in culture medium was affected by cisplatin in a similar pattern (Fig. 2). The activity of γ-GT in culture medium was not changed by cisplatin treatment for 6 hours upto 1mM of concentration. Treatment with 50μM and 100μM of cisplatin for 24 hours markedly increased (7-fold) γ-GT activity in media, but treatment with 500μM of cisplatin reduced these values to control level. The activity of γ-GT in media was significantly enhanced to 500% and 330% of control by treatment with 50 and 100μM of cisplatin for 48 hours, respectively. Above concentration of 100μM, γ-GT activity in media was also decreased to control value.

In the recent past the *in vitro* measurement of the leakage of the cytosolic enzyme LDH from isolated kidney cell[17] or renal cortical slices[18] has been used as a method to determine cell viability and the extent of the toxic effect of substances such as cisplatin. In this study activities of LDH and γ-GT in culture medium were significantly increased by treatment with cisplatin for 24 and 48 hours from 50μM to 100μM of concentration, but above this concentration, the activities of these enzymes in media were markedly decreased to control levels, despite of increase of cytotoxicity. This result indicates that cisplatin has direct inhibitory effect on activities of these enzymes. Cisplatin is known to inhibit the activities of many enzymes,[19,20,21] especially sulfhydryl-group containing enzymes. Inhibitory effect of cisplatin on LDH activity seems not to be related to the nephrotoxicity of cisplatin, because nontoxic platinum compound, transplatin also had an severe inhibitory effect on activity of LDH.[22] These results make it doubtful to use enzyme leakage experiments to demonstrate damage of kidney cells by chemicals such as cisplatin.

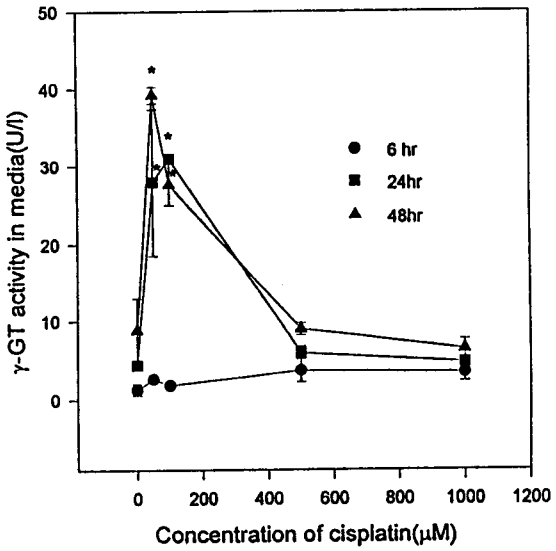

Fig. 2. γ-Glutamyltransferase (γ-GT) release by LLC-PK$_1$ cells treated with different concentrations of cisplatin for 6, 24, 48 hours. Values are expressed by means±SD of three seperate experiments. *Significantly different from the control at $p<0.05$.

2. Effect of Cisplatin on α-Methylglucose Uptake

In order to test the uptake of α-MG as a nephrotoxic parameter three nephrotoxicants, cisplatin, mercuric chloride and cadmium chloride were used. The cytostatic drug, cisplatin

treatment produced time- and dose-dependent inhibition of α-MG uptake. Treatment with 25μM and 50μM of cisplatin for 6 hours caused significant inhibition of α-MG uptake (68% and 53% of control) (Fig. 3). Treatment with cisplatin for 24 hours caused marked decrease in α-MG uptake at concentration of 5μM (Fig. 4). α-MG uptake was inhibited to 50% of control by treatment with 25μM of cisplatin for 24 hours.

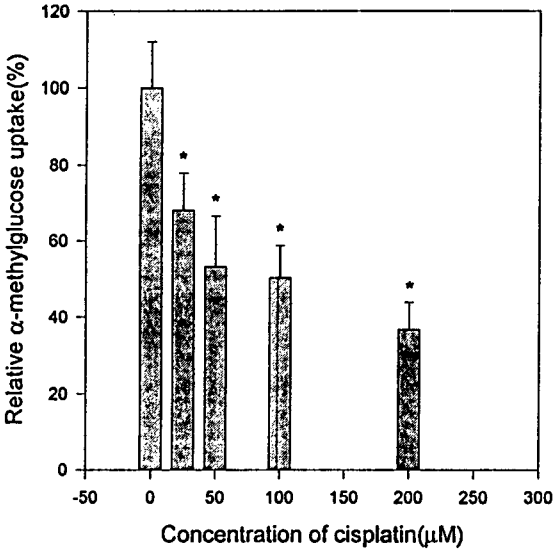

Fig. 3. Effect of cisplatin on cellular integrity as assessed by α-MG uptake. Cells were incubated at various concentrations of cisplatin for 6 hours before assessment of α-MG uptake. Values are expressed by means±SD of three seperate experiments. *Significantly different from the control at $p<0.05$.

α-MG proved to be a very suitable substrate to assess the transport capacity of the LLC-PK 1 cells: most xenobiotics known to produce damage to proximal tubular cells *in vivo* caused loss of cell function *in vitro* too, as could be sensitively detected by measuring the ability to take up α-MG. α-Methylglucose is actively taken up by the proximal tubular cell via the apical Na^+/

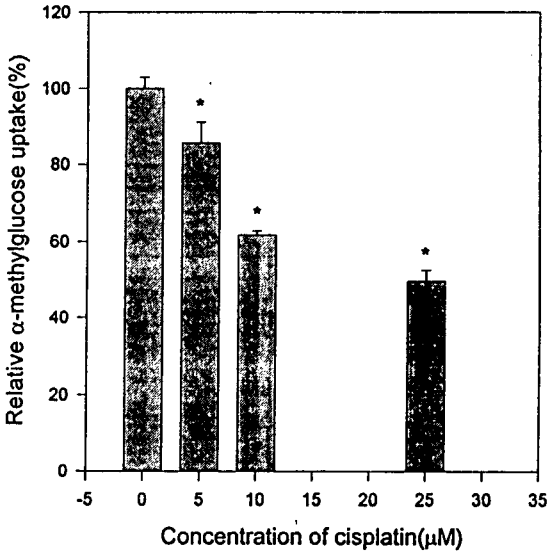

Fig. 4. Effect of cisplatin on cellular integrity as assessed by α-MG uptake. Cells were incubated at various concentrations of cisplatin for 24 hours before assessment of α-MG uptake. Values are expressed by means±SD of three seperate experiments. *Significantly different from the control at $p<0.05$.

glucose cotransporter, which is driven by the Na^+/K^+-ATPase in the basolateral membrane. The activity of the Na^+/K^+-ATPase is dependent on the intracellular ATP concentration, which, in turn, depends on mitochondrial activity (Fig. 5). If any of these processes are disturbed, the α-MG uptake will be affected. A toxic compound may, thus, inhibit α-MG uptake independently from the ATP content of the cell: it may affect the Na^+/glucose cotransporter and/or the Na^+/K^+-ATPase. Alternatively, inhibition of the α-MG uptake may be mediated by an ATP shortage due to a disturbance of mitochondrial function caused by either a functional damage or a limited substrate availability.

In this study, cisplatin inhibited uptake of α-MG at concentrations which renal enzymes were not leaked. This result agrees with the fact that LDH leakage is an endpoint determination,

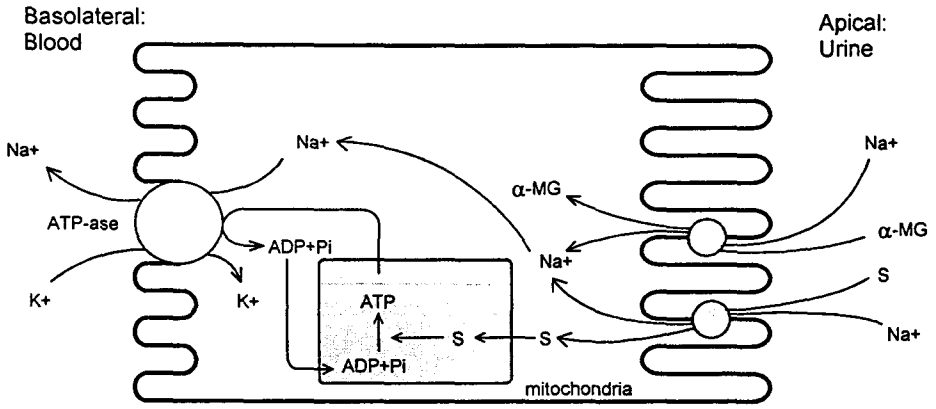

Fig. 5. Schematic representation of a proximal tubular cell, indicating functional relationships between intracellular ATP concentration and α-MG uptake. ATP is the driving force for the Na^+/K^+-ATPase which creates a sodium gradient across the cell membrane, allowing Na^+/glucose transport to proceed as a secondary active transport process. The intracellular ATP concentration depends on the oxidative phosphorylation in the mitochondria, which is dependent on functional integrity of this system and substrate (S) availability.

indicating cell death, while α-MG uptake assesses loss of a specific cellular function, which precedes cell death. A loss of α-MG transporting capacity does not immediately lead to cell death. However, although glucose uptake may not be immediately critical for the viability of the proximal tubular cell, it reflects an essential function of these cells. Therefore, any disturbance may serve as an early indicator of cell toxicity. This inhibitory effect of cisplatin on the α-MG uptake most likely does not represent damage to the glucose transporter directly since in brush border membrane vesicles cisplatin was shown to have no effect on D-glucose transport.[23] Inhibitory effect of cisplatin on α-MG uptake may be due to inhibition of Na^+/K^+-ATPase activity or decrease in intracellular ATP content. It has been shown that cisplatin had no effect on activity of Na^+/K^+-ATPase up to concentration of 33μM in cultured rabbit tubular cells.[24] Thus, inhibition of α-MG uptake by cisplatin can be produced by the mitochondrial dysfunction resulting in a decrease of intracellular ATP content. The effect of cisplatin on α-MG uptake could be detected, even at a concentration as low as 10μM. Concentration of 10 μM cisplatin which inhibits α-MG uptake can easily be attained in the kidney even at a relatively low dose of 5mg/kg.[25] Although acute renal failure develops only several days after administration, histophathological damage is observed within a few hours,[15] and the extent of ultimate renal damage is dependent on factors occurring during or immediately after cisplatin administration.[26]

3. Effect of $HgCl_2$ and $CdCl_2$ on α-Methylglucose Uptake

Incubation of the cells with 10μM mercuric chloride for 6 hours produced significant inhibition of α-MG uptake (Fig. 6). Mercuric chloride markedly decreased α-MG uptake to 45% of control at concentration of 25μM for 6 hours treatment. Mercuric chloride inhibited the uptake of α-MG very severely, even at very low concentration. This may be due to binding of Hg

$^{2+}$ to an essential sulfhydryl group of the glucose carrier, thus inhibiting transport was produced immediately and severely. Alternatively, Hg^{2+} could affect the Na^+/K^+-ATPase activity resulting in a loss of the Na^+ gradient over the cell membrane which is necessary for α-MG transport, thus causing a secondary loss of α-MG uptake. Additional information on the exact intracellular site of the nephrotoxic action of Hg^{2+} may be obtained by measuring the intracellular K^+ concentration (Na^+/K^+-ATPase activity) and O_2 consumption (mitochondrial capacity for ATP synthesis).

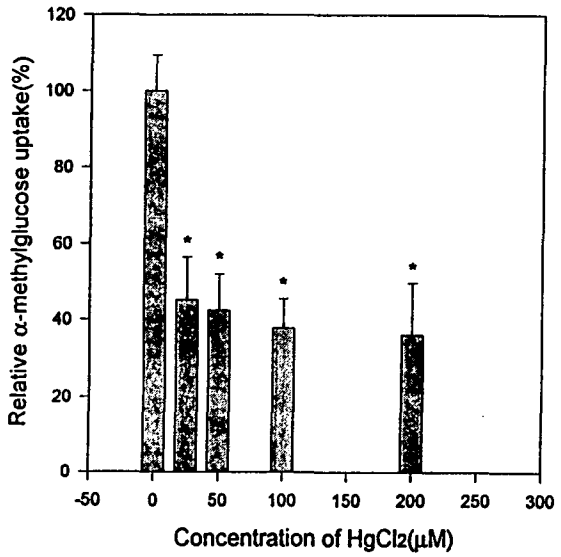

Fig. 6. Effect of $HgCl_2$ on cellular integrity as assessed by α-MG uptake. Cells were incubated at various concentrations of $HgCl_2$ for 6 hours before assessment of α-MG uptake. Values are expressed by means±SD of three seperate experiments. *Significantly different from the control at $p<0.05$.

Cadmium chloride was also able to inhibit α-MG uptake (Fig. 7). Treatment with 25μM of cadmium chloride reduced α-MG uptake, but this difference was not significant. Cadmium

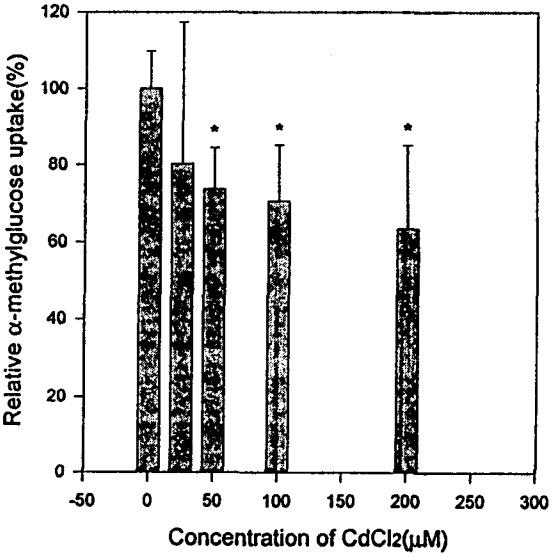

Fig. 7. Effect of $CdCl_2$ on cellular integrity as assessed by α-MG uptake. Cells were incubated at various concentrations of $CdCl_2$ for 6 hours before assessment of α-MG uptake. Values are expressed by means±SD of three seperate experiments. *Significantly different from the control at $p<0.05$.

chloride produced significant inhibition of α-MG uptake (73.6% of control) at 50μM of concentration for 6 hours treatment. Cadmium as an environmental pollutant has aroused a great concern due to its toxic effects on various body tissues.[27] Liver and kidney of humans are reported to contain about 50% of the total body burden of Cd.[28] In experimental animals also, more than half of the absorbed Cd accumulated in the liver and kidney with the evidence of histological and functional damage.[29,30,31] *In vivo* it is difficult to prove that acute nephrotoxic effect of Cd^{2+} are due exclusively to direct nephrotoxic because observed renal disturbances might be due to damage inflicted on other organs, in particular the liver.[32]

Among the tested metals, mercuric chloride proved to be the most toxic metal, and cadmium

chloride to be the least toxic metal.

CONCLUSION

This study shows that LLC-PK$_1$ cells are suited for studies on mechanisms of toxic insults toward the proximal tubules because this cell line contains relatively high levels of proximal tubule marker enzymes and possesses a Na^+-dependent active sugar transport system similar to that found in proximal tubules. Compared to determination of leakage of enzymes *in vitro* systems, α-methylglucose uptake is a sensitive and rapid parameter which assesses damage to cell function at an early time point. Additional mechanistic information, indicating the intracellular site of nephrotoxic action, can be obtained by coupling the α-MG and ATP data to measurements of intracellular K^+ concentrations and/or O_2 consumption under various conditions.

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