Proteomic Analyses of Contrast Media –Treated Mesangial Cell

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Abstract: Contrast Media is used in angiography, urography and tomography. It has been reported that contrast media nephrotoxicity incidence is 50% higher in patients with diabetic nephropathy undergoing coronary angiography and approximately 15% of these patients developed renal failure. Proteomic analyses are a promising tool to study renal pathophysiology. In the future identification of biomarkers in renal diseases will develop therapeutic targets that will decrease the damage of acute renal failure. To identify proteins that were up or down-regulated in immortalized mesangial cells in a culture in the presence of contrast media we evaluated three groups: control, manitol used as control of osmolarity and Hexabrix™. The cell homogenate from each group was submitted to the 2D-PAGE analysis, and proteins were identified by MALDI-TOF mass spectrometry. Some proteins were expressed only in the Hexabrix group (Proliferating Cell Nuclear Antigen and Masp 1); others were up-regulated in the Manitol group (Translationally Controlled Tumor Protein), a protein was expressed only in the Control and Manitol (Annexin A3) groups, and we identified proteins that were expressed in all three groups (Heat shock proteins 27 and 84, protein disulfide isomerase A3 precursor and Beta-actin 1). We believe that other substances present in Hexabrix, possibly the iodine, could be the regulatory factor of these proteins and the Hexabrix could be responsible for the nephrotoxicity observed in these cells.

Key Words: Acute renal failure, mesangial cell, nephrotoxicity, contrast media, 2-DE, proteomics, mass spectrometry.

INTRODUCTION

The major roles of the kidney are blood filtration which takes place in the glomerulus, and also homeostasis, maintained by the selective reabsorption of water and eletrolites. The functioning unit of the Kidney is the nephron which consists of the glomerulus, proximal and distal tubules and Henle´s loop. The glomeruli are composed of mesangium and have three types of cells: epithelial, endothelial and mesangial cell (MC).

There are two types of MC: one which has a contraction capacity and regulates the filtration surface area and has the ability to synthesize bioactive products and the other originates from bone marrow and has phagocyting properties. It is now apparent that CM induces reduction in renal function predominantly through modulating tubular regulatory mechanisms and production of renal vasoactives autacoids [1].

MC are distinguished from other kidney cells by their ability to synthesize a great number of substances like growth factors, vasoactive agents such as cytokines, tyrosine hydroxylase, catecholamines, angiotensin I-converting enzyme (ACE), renin, prostaglandins [2-6] and neutral endopeptidase (NEP) [7-10].

The kidney is a common target organ for toxic agents such as contrast media (CtM). CtM with iodine is used in angiography, urography and tomography. This substance is incorporated intact into the cells and it is freely filtrated and metabolized in the kidney. After CtM incorporation by mesangial cells, the iodine concentration is significantly higher in both the nuclei and cytoplasm [11]. Portier *et al.* [12] in 1997 observed that MC exposed to 90 and 100 mg Iodine/mL of ioxaglate resulted in 61% and 65% of cell death, due to its cytotoxic effects. CtM in a final concentration of 0.1% induced apoptosis in cells, probably due to a significant impact on the cytokine production thus eventually affecting renal response to the injury [13].

Berg [1] described that a broad use of ionic and non ionic contrast media of high and low osmolarity was the third greatest cause of acute renal failure (ARF). Although Nicot *et al.* [14] found that renal arteriography using CtM can be followed by persistent or transient proteinuria without significant changes in renal function; it is already known that it may cause glomeruli hemodynamic alterations and induction of MC or glomeruli contractions [15].

Previous studies by our group and others confirmed a reduction of around 50% in the concentration of Angiotensin I (Ang I) and a two fold increase in the concentration of Angiotensin II (Ang II) in MC exposed to ioxaglate of sodium meglumine contrast medium (Hexabrix™), suggesting that the CtM induces the activation of Angiotensin converting enzyme to convert Ang I to Ang II. This data is supported by the fact that Ang II and endothelin are increased in the circulation after the administration of CtM causing an augmentation of vasoconstriction and a medullar ischemia that are responsible for the induction of ARF [2, 16-19].

Contrast media nephrotoxicity (CMN) is lower than 1% in healthy patients; however a 50% higher incidence has been reported in patients with diabetic nephropathy undergo-

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ing coronary angiography and approximately 15% of these patients developed renal failure [20].

In general any pathological state that decreases renal function represents a high risk of nephrotoxicity induced by CtM [21]. In patients presenting with conditions such as diabetes, dehydration, hypertension, renal insufficiency, aged over 60 years, diabetic nephropathy, heart failure, concomitant exposure to other nephrotoxins are more susceptible to ARF caused by CtM [12, 22].

The mechanisms of the pathogenesis of CtM are complex and not fully understood. The main factors are osmotic, toxic and hemodynamic [23]. The osmotic effects could be explained by the high osmolarity of urine after CtM injection that creates an increase in intrabubular hydrostatic pressure that decreases the filtration pressure in glomeruli and glomerular filtration rate. Sodium and water excretion increase markedly as a consequence of osmotic diuresis. An increased load of sodium to macula densa in the distal tubule stimulates the tubuloglomerular feedback mechanism, which leads to a decrease in glomerular filtration rate [1]. The osmotic diuresis and the increased delivery of salt to the distal nephron induced by CtM increase renal metabolic activity and renal oxygen consumption and aggravate medulary hypoxia [24].

As a direct toxic effect of CtM we could detect vacuolization of the proximal tubular cells. Quantitative determination of proximal tubular enzymes in urine has proven extremely sensitive in the evaluation of CtM nephrotoxicity [23]. However, some studies concluded that the effect of CtM on urinary enzymes cannot be explained only by the osmotic load and is probably caused by iodine or other specific CtM related factors [1].

Contrast induced renal vasoconstriction has been identified as one of the main mechanisms of ARF being an example of hemodynamic effects. The factors involved in vasoconstriction are changes in intrarenal intracapsular pressure, direct effects of CtM on smooth muscle contractility through alterations in intracellular hydration, initial vasodilatation and alterations in intracellular calcium concentration [25].

Today, many proteins can be analyzed at the same time, using the two-dimensional gel electrophoresis (2-D) a powerful and widely used research tool in proteomics. This method consists of consecutive separation based in different properties of proteins. The first dimension called isoelectrofocusing (IEF) separates proteins according to their isoelectric points (p*I*). The second dimension separates them according to their molecular weights (M_r) using polyacrilamide gel electrophoresis. This process prepares proteins that can be analyzed by peptide mass fingerprint protein identification *via* matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Maldi-TOF MS) [26].

The advantage of the proteomic analysis is the potential discovery of biomarkers that can be used as indicators of toxicity or for therapeutic purposes. The main goal of the present paper is to evaluate the chemotoxic effects of iodinated radiological contrast media Hexabrix^{TM} 320 on kidney cells, identifying the proteins that were up or down regulated in the presence of Hx which could be involved in the mechanisms that cause nephrotoxicity. This information could be used in the future for diagnostic purposes and also in treatments that could minimize renal injury caused by contrast media.

MATERIALS AND METHODOLOGY

Immortalized Mesangial Cells Culture (IMC)

Cells were purchased from the American Type Culture Collection (ATCC: CRL-1927). Mouse IMC and cultured in DMEM (Gibco BRL, USA) supplemented with 5% fetal bovine serum (FBS), 50 U/mL penicillin and 6 mg/mL streptomycin (Sigma Co, USA). Incubations were carried out in a humidified atmosphere of 5 % CO_2 in air at 37°C.

When the cells achieved 100 % confluence, the cultures were submitted to trypsinization and subcultured in plates under the same culture conditions.

Treatment of IMC

In order to evaluate the effects of CtM we used two experimental groups where cells were treated with an equal concentration of 4.8 mg/ml Man or Hx (Guerbet Laboratory, France) diluted in the serum-free DMEM to give the same final osmolarity (600mOsm/kg of water) and one control group (Ct) where IMC received only serum-free DMEM. IMC were cultured at 37°C for 6 hours in a 5% CO_2 humidifier incubator. The final concentration being 4.8 mg/mL. This concentration for manitol and hexabrix was determined based on the doses used on humans in the exams that were 20 to 100 mL of contrast media or 6400 a 32000 mg of iodine, equivalent to 0.0094 to 0.4571 mg iodine/ mg weight. The dose of 4.8 mg/mL in our experiments corresponds to 0.048 mg iodine/mg of weight, this being the concentration level used in the clinical practice daily.

Cell Viability

The cell viability was evaluated using two fluorescent stains using 10 μL a solution containing 100 μg/mL of orange acridine (OA) and 0.3 μL of ethidium bromide (EB) which were added to the cell suspension. The cells from the three groups were submitted to this test and under observed a fluorescence microscope where green cells were considered viable and orange ones, not viable. Viability is given in percentage (%) of viable cells in the total sample.

Cell Preparation

After incubation, the cells were rinsed twice with phosphate-buffered saline (PBS), scraped in 2 mL of PBS and centrifuged at 800 g for 10 min at 4℃. The supernatant was discarded and the cell pellet was collected and stored at -80^{o}C for later assays.

The pellet was suspended in 1.0 mL of sample buffer consisting of 8 M urea, 1 mM EDTA (Merck, Germany), 2% Triton X-100 (Sigma Co, USA) 0,01 mM DTT (Invitrogen, New Zealand), 0.5 % IPG buffer 3-10, (GE Healthcare/ Amersham Biosciences, Sweden) and was sonicated for 30 secs and centrifuged at 10,000 g for five minutes, at -4^{o}C. The supernatant was concentrated by filtration using a centricon centrifugal filter (Millipore, EUA) with ultra filtration membrane of 10 kDa cut off limit and lyophilized in a Speed Vac system (Savant, USA).

Protein Quantification

Cellular homogenate protein concentration was determined by the Lowry method using DC-Protein kit (Bioagency, Brazil) and bovine albumin as standard, according to the manufacturer's recommendations.

Two-Dimensional Electrophoresis (2-D)

For the isoelectrofocusing 500 µg of protein was loaded onto 13 cm Immobiline DryStrip (pH 3-10) (GE Healthcare/ Amersham Biosciences, Sweden). Focusing started at 200 V and the voltage was gradually increased to 8.000 V and then kept constant for 4 hours. After the first dimension, the strips were equilibrated for 15 min in a buffer containing 8 M urea, 50 mM Tris-Cl pH 8.8, 30 % glycerol, 2 % SDS, 2 % DDT, and afterwards for 15 minutes in the same buffer containing 3% iodocetamide in place of DDT. After equilibration, the strips were loaded onto the top of a 10% gradient sodium dodecylsulfate polyacrilamide gel (SDS-PAGE) for the second dimension separation, which was carried out on 50 mA/gel for 6 hours using Dual Color (Bio-Rad, USA) as a molecular mass standard (25-250 kDa). Immediately after the second dimension the gels were stained overnight with Coomassie Blue (Bio-Rad, EUA) on a rocking shaker, and they were analyzed in a GS-710 Calibrated Imaging Densitometer (Software PDQuest 7.0, Bio-Rad, EUA). In PDQuest we visualized a total os spots in each gels: 35 spots in Ct, 70 spots in Man and 26 spots in Hx groups. From these 11 spots in CT, 17 spots in Man and 20 spots in Hx groups showed altered level of expression.

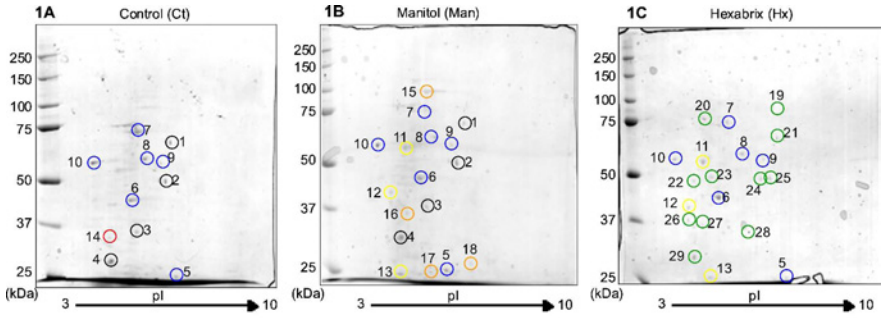
Analyses expressions were between Ct and treatment groups. We identified 8 spots down-regulated and 17 spots up-regulated in cell treated with Hx. In Man treatment 3 spots were down-regulated and 15 spots up-regulated.

Matrix-Assisted Laser Desorption Ionisation Mass Spectrometry (MS)

Spots were automatically excised from the gels using the Xcise™ spot picker (Shimadzu Biotech, Japan) and destained in a solution of 100 mM ammonium bicarbonate and 50% acetonitrile in proportion 1:1. Digestion was performed in 30 µL of a solution containing 10 ng/µL trypsin diluted in 25 mM ammonium bicarbonate, overnight at 30°C. After incubation, aliquots of 0.5 µl of the samples were prepared in a matrix solution containing α-cyano-4 hydroxy-cinnamic acid (CHCA) and were applied on the slide with a matrix thinlayer (10 mg/mL/70% acetonitrile / 0.1% TFA). The samples were allowed to air dry at room temperature. The mass spectra were obtained using the AXIMA-CFR plus™ (Shimadzu Biotech, Japan) Maldi-TOF/MS. Peptide mass profiles produced by Maldi-Tof were analyzed using Mascot™ software (Matrix Science, UK). Peptides masses were compared with the theoretical masses derived from sequences contained in SWISS-PROT and NCBInr data banks. We used search parameters as follows: monoisotopic masses, with a tolerance of 0.1-0.5Da, one missed tryptic cleavage, a carbamidomethyl modification of cysteine and variable modification of oxidation of methionine.

RESULTS

The cells viability in the presence of Man and Hx was approx. 97 %. Fig. (**1A**, **1B** and **1C**) respectively show 2-D maps generated for IMC Ct, IMC submitted to Man and to Hx and we can see the protein spots that were visible in one, two or in the three gels obtained. Fig. (**1D**) is a representation of the presence/absence of the spots in the gels. We observed a higher concentration of acidic proteins with molecular mass ranging from 37 to 100 kDa for all treatments. Cells



1D	Ct	Man	Hx	Total
RED	+	-	-	1
BLACK	+	+	-	4
BLUE	+	+	+	6
YELLOW	-	+	+	3
ORANGE	-	+	-	4
GREEN	-	-	+	11

Fig. (1). 2-D maps of IMC Control (**1A**), Manitol (**1B**) and Hexabrix ™ (**1C**). Proteins were extracted and separated on a pH 3–10 linear IPG strip, followed by a 10% PAGE, using molecular weight markers from 25-250 kDa, as described in Methods. After gel electrophoresis the gels were Coomassie stained, scanned and the images analyzed. The visualized spots (circle) were analyzed by MALDI-MS. In (**1D**) is a representation of the presence/absence of the spots in the gels.

incubated with Hx expressed 11 proteins that were not found in the other groups.

Fig. (**2**) shows the comparison of the gels and all the protein spots which differed significantly in the expression level between the treatments zoomed in. The proteins were identified by MALDI-TOF MS.

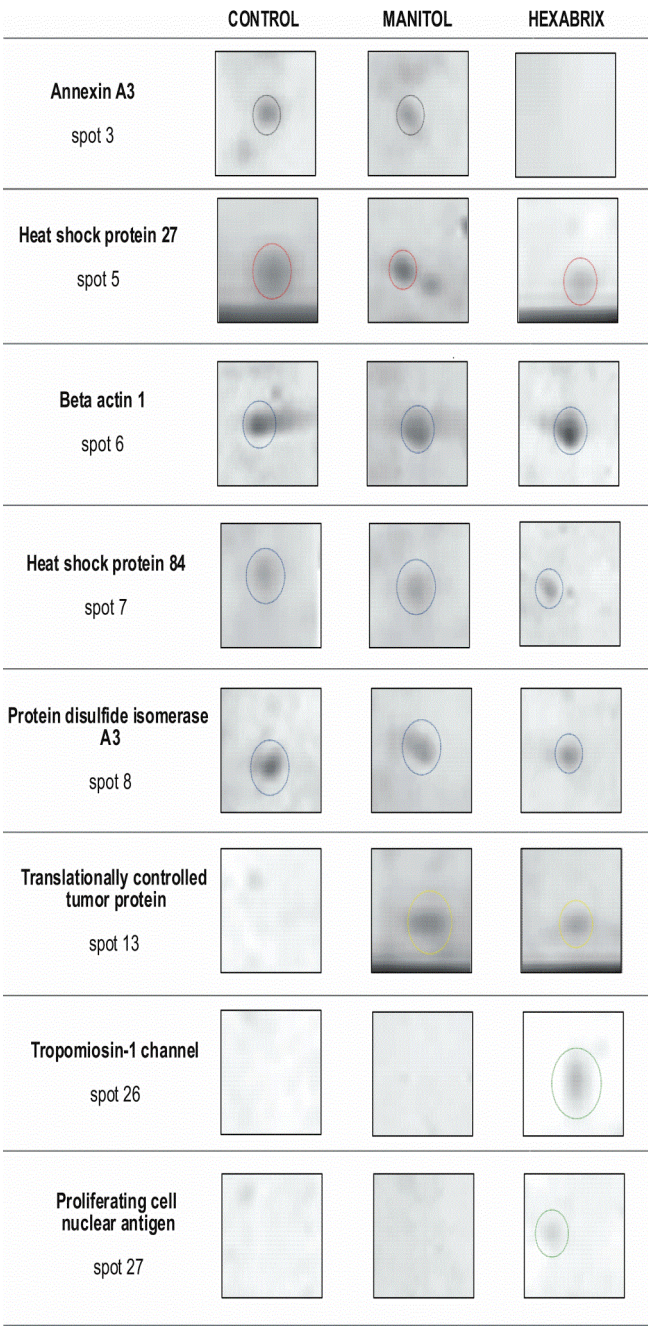


Fig. (2). Comparison between maps of IMC submitted to the treatments: Control, Manitol and Hexabrix. All protein spots in the 2-D gels, which differed significantly in the expression level between the treatments and the proteins were identified by MALDI-TOF MS, are zoomed in. The spot numbers are the same as shown in Fig (**1**).

The pixels analyses of each of the Coomassie gel spots (n = 3) permitted the statistical analysis ("One Way ANOVA") of proteins and showed that the protein expression of spots 5, 11 and 13 of Man gel were statistically higher than that of Ct or Hx. The protein in spot 12 was up-regulated in Hx. The other spots did not present statistically significant differences but some of them disappeared in the presence of the Hx or were absent in the Control gel (Fig. **3**).

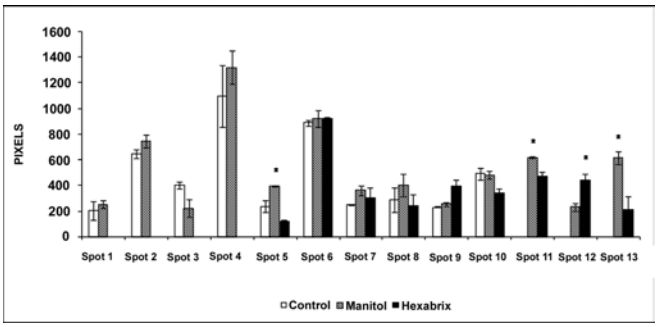


Fig. (3). Bar Graphs with densitometric analyses of protein spots in pixels present in two or three gels. The spot numbers are the same as shown in Fig. (**1**). The proteins represented by the spots labeled with (*) differed significantly in the expression level between the treatments. The values are average ± SE, n = 3, (*$p < 0.05$) from "One way ANOVA".

Spots of interest were excised, distained, digested and analyzed by mass spectrometry (MS). In Table **1** we show a list of spots as being variable and proteins identified by MALDI-TOF MS.

DISCUSSION

Contrast media is a substance that interacts with the kidney specifically in mesangial cells. All the radiocontrast substances cause glomeruli hemodynamic alterations such as decline in the filtration rate and an increase of medullar hypoxia. Iodine Contrast Media can be a cause of ARF in patients with diabetes, dehydration, hypertension, renal insufficiency, aged over 60, heart failure and exposure to other nephrotoxins [21, 22].

The CtM mechanism of action in the kidney is still not very clear despite some clinical studies, but we believe that the toxic effect of the CtM can modify renal protein expressions causing injury.

In this study we used gels and mass spectrometry to identify the proteins. This approach has not been previously used in the analysis of the effects of contrast media in the IMC.

We analyzed the protein expression in the Ct, Man and Hx groups, where we detected many of them were up or down regulated in the different groups. The majority of the proteins were acidic with a molecular mass range from 37 to 100 kDa in the three studied groups. After 2-D we identified some proteins that were present only in the Hx gel such as: Tropomyosin-1 channel (TPMA) and proliferating cell nuclear antigen (PCNA).

The TPMA is a protein located in cytoskeleton that binds to actin filaments blocking actin and myosin interaction, causing changes in its organization. The presence of this protein indicates that CtM may directly affect the actin filaments causing alterations in the glomerular hemodynamics. A similar result was described by Brezis *et al*. [15] which

Table 1. List of Spots as Being Variable and Proteins Identified* by MALDI-TOF MS

Spot No. [a]	Protein Name	Number from NCBI Database	Mr/pI [b] Experimental (Theoretical)	Score [c] (p<0.05)	Coverage %	Matched Peptides	Mass Error Tolerance (ppm)	Control *(pixels)*	Manitol *(pixels)*	Hexabrix™ *(pixels)*
1	----------	----------	----------	--------	----------	----------	----------	205	252	0
2	----------	----------	----------	--------	----------	----------	----------	647	745	0
3	Annexin A3	gi 7304887	42.4/5.5 (36.34/5.3)	135	33	12	126	404	222	0
4	----------	----------	----------	--------	----------	----------	----------	1098	1322	0
5	HSP 27 / HSPB 1	gi 17390597	25.1/6.0 (23.0/6.1)	70	40	8	71	239	394	123
6	Beta-actin 1	gi 49868	39.8/4.9 (39.16/5.0)	79	21	8	42	891	924	925
7	HSP 84	gi 40556608	83.0/5.1 (83.29/5.3)	114	24	14	27	251	360	308
8	Erp57 / Protein disulfide isomerase	gi 23958822	53.8/5.4 (56.64/5.8)	90	24	13	78	286	399	243
9	----------	----------	----------	--------	----------	----------	----------	232	254	394
10	----------	----------	----------	--------	----------	----------	----------	492	481	346
11	----------	----------	----------	--------	----------	----------	----------	0	618	475
12	----------	----------	----------	--------	----------	----------	----------	0	230	439
13	Translationally controlled tumor protein	gi 30385204	23.1/4.7 (19.45/4.7)	85	29	8	62	0	614	210
14	----------	----------	----------	--------	----------	----------	----------	454	0	0
15	----------	----------	----------	--------	----------	----------	----------	0	356	0
16	----------	----------	----------	--------	----------	----------	----------	0	351	0
17	----------	----------	----------	--------	----------	----------	----------	0	319	0
18	----------	----------	----------	--------	----------	----------	----------	0	339	0
19	----------	----------	----------	--------	----------	----------	----------	0	0	203
20	----------	----------	----------	--------	----------	----------	----------	0	0	267
21	----------	----------	----------	--------	----------	----------	----------	0	0	412
22	----------	----------	----------	--------	----------	----------	----------	0	0	302
23	----------	----------	----------	--------	----------	----------	----------	0	0	205
24	----------	----------	----------	--------	----------	----------	----------	0	0	327
25	----------	----------	----------	--------	----------	----------	----------	0	0	287
26	Tropomiosin-1 channel (TPMA)	gi 509182	37.1/4.7 (32.6/4.6)	71	22	16	56	0	0	318
27	Proliferating cell nuclear antigen (PCNA)	gi 13543223	28.5/4.7 (28.9/4.6)	70	20	7	104	0	0	245
28	----------	----------	----------	--------	----------	----------	----------	0	0	322
29	----------	----------	----------	--------	----------	----------	----------	0	0	447

*The following criteria were used to consider a spot as being variable: i) consistently present or absent in all three replicates; ii) ratios between treatments higher than 5-fold, indicates quantitative differences statistically significant (*Anova* $p<0.05$).

a. As indicated in Fig. (**1**); b. Calculated with standard molecular weight markers (*Mr*) and (p*I*) on PDQuest software; c. MASCOT score >69 corresponding to $p<0.05$.

suggested that the iodine or other substances in CtM could act directly in the actin filaments in MC.

We suggest that the Hx stimulates an inflammatory reaction since we observed PCNA only in the cells that were treated with this drug. The presence of this protein induces interleukin-2 production stimulating the proliferation of T-lymphocytes being responsible for the inflammatory response. Studies showed that the PCNA expression in renal cells is related to renal disease progression [27].

The up-regulation of this protein in glomeruli and renal cortex from focal segmental glomerulosclerosis patients has already been described by Geleilete *et al*. [27].

Other proteins were present in the Man and Hx groups and were absent in the control gels e.g. the translationally controled tumor protein (TCTP) that has an anti-apoptotic function. This data is in agreement with the results reported by Peer *et al*. [13] where they observed an increase in IMC apoptosis even when submitted to low CtM concentration due to alterations in the quality and production of cytokines. We believe that in our study the osmolarity was responsible for the up-regulation of TCTP, since the osmotic concentration of both treatments was equal and it differs from the control group.

On the other hand, an protein was expressed only in the Ct and Man groups such as annexin A3 suggesting that the Hx was responsible for the down-regulation of these proteins probably due to its iodine content.

Annexin A3 participates in the regulation of membrane organization and traffic and in the regulation of Ca^{2+} currents across membranes or Ca^{2+} concentration within the cells. The Ca^{2+} acts as a secondary messenger in a variety of processes which couple extracellular signals to cellular responses. Moreover, this protein acts like a phospholipase A2 inhibitor. The cells treated with Hx did not express this protein, thus we suggest that phospholipase A2 is not inhibited being able to produce inflammatory and vasoconstrictor hormones that could cause the ARF.

Finally, we identified proteins that were expressed in the three groups Heat shock proteins 27 (Hsp27) and 84 (Hsp84), Beta actin-1 and protein disulfide-isomerase A3 precursor (ERp57).

In conclusion, we believe that other substances present in Hx, possibly the iodine, could be the regulatory factor of these proteins. This hypothesis is supported by the results shown by Nordby *et al*., 1989, where significant iodine concentrations had been found in the cytoplasm and the nucleus of mesangial cells treated with Hx [11].

Thus we suggest that the CtM osmolarity could be regulating the synthesis and/or expression of mesangial cell proteins. However, the molecular pathways leading to the cell injury induced by radiocontrast cytotoxicity are still obscure.

These data generated new questions on the function of proteins identified here. Proteomics expression alone does not provide any functional or physiological significance. Further studies are necessary to understand the physiological role of the identifield proteins another possible mechanism involved the down or up regulation of these proteins. Our data suggests that the radiocontrast agent could have a chemical/molecular toxicity beyond their physicochemical effects.

These findings could be helpful in the diagnosis and the treatment of patients presenting Hexabrix^{TM} toxicity. Moreover, proteomic analysis is a promising tool to study renal pathophysiology that could lead in the future to the identification of biomarkers of renal diseases which could be therapeutic targets minimizing the actual damage of acute renal failure.

CONCLUSION

We believe that other substances present in Hx, possibly the iodine, could be the regulatory factor of these proteins and the Hx could be responsible for the nephrotoxicity observed in these cells.

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