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Protective Potency of Nano-emulsified Amifostine against Cisplatin Induced Nephrotoxicity in Rats

REHAB A. MOHAMED*, FATMA F. ABDEL HAMID[1], DAWOUD F. HABIB, NADIA A. MOHAMED, WAGDY K. B. KHALIL[2], EMAD K. AHMED[1] AND AMANI F. H. NOURELDEEN[1]

Medical Biochemistry Department, National Research Centre, Dokki, Giza, Egypt
**(e-mail : roroanas30@yahoo.com; Mobile : +200 12231 19879)*

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ABSTRACT

Drug-induced kidney injury is one of the causatives of acute kidney failure. Amifostine loaded silica nano emulsion was synthesized using water/oil emulsion with the help of ultra-sonication waves. gp1 normal control (NC) injected with saline daily for one month, gp2 injected with particles of silica nano emulsion (SiNPs) in a dose of 150 mg/kg/day for one month, gp 3 injected twice with cisplatin in a dose of 20 mg/kg body weight to induce nephrotoxcity, gp 4 injected with particles of silica nano emulsion of amifostine (SiNPs@AMF) in a dose of 150 mg/kg/day for one month and gp 5 injected with particles of silica nanoemulsion of amifostine (SiNPs@AMF) in dose of 150 mg/kg/day for three weeks then injected with two doses of (cis) (20 mg/kg, day after day) followed by amifostine nanoemulsion injection to complete one month**.** Results demonstrated that HPLC analysis of serum homocysteine and tissue inhibitor metalloproteinase-1 (TIMP-1), osteopontin and RANTS levels showed a significant elevation in Cis group in comparison with NC group and down regulation of the expression of nephrin mRNA and podocin mRNA strongly in nephrotoxic group. Nephrotoxic animals protected with amifostine nanoemulsion recorded a less significant increase in serum homocysteine, tissue inhibitor metalloproteinase-1 (TIMP-1), osteopontin and (RANTS) levels with enhancement expression of nephrin mRNA and podocin mRNA compared to NC.

Key words: Podocyte, nephrotoxicity, silica nanoemulsion, amifostine, cisplatin, nephrin

INTRODUCTION

Drug-induced nephrotoxicity is closely linked with acute renal damage and chronic kidney diseases (Sales *et al.*, 2020). Proteinuria is a marker of renal disorders and shows a poor prognosis. Proteinuria is also considered a pivotal risk factor for the progression of renal diseases (Wang *et al.*, 2019) .The dysfunctions of the glomerular filtered tospacious leakage of plasma proteins and diffuse alteration of podocyte foot processes (detected by electron microscopy), was observed in many forms of nephropathy or glomerulonephritis. Heavy proteinuria is the cause of the nephritic syndrome (Menon, 2019).

The molecular processes implied glomerular diseases with proteinuria are not fully understood, even though elevating evidence indicates a key role for podocytes in permeability changes at the glomerular filtration barrier. Experimental animal models of glomerulonephritis have shown a correlation between the alteration of nephrin expression and proteinuria (VanDeVoorde, 2015), thus, the nephrin expression was studied in certain acquired kidney diseases (Kaveripakam and Adikay, 2017).

Amifostine (AMF) is an organic thiophosphate antioxidant used for the protection of normal tissues from chemotherapeutic or radiotherapy drugs in cancer patients (Deng *et al.*, 2020). The biological activity is relying on its hydrolysis by alkaline phosphatase into the active metabolite with sulfhydryl. The sulfhydryl could scavenge oxygen-derived free radicals to prevent DNA damage (Valko *et al.*, 2016). The cytoprotective selectivity of AMF is mainly related to the diversity in extracellular acidity and alkaline phosphatase expression between normal tissue and tumor (Xiao *et al.*, 2016). The active metabolite of AMF has very limited stability *in vivo* because of the rapid formation of disulfide from free sulfhydryl, as

[1]Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt.
[2]Cell Biology Department, National Research Centre, Dokki, Giza, Egypt.

such the high dose is needed to realize eligible protective effects. Clinically, increase drug concentration in blood results into reverse effects as vomiting, hypotension and nausea, which has significantly limited the extensive application of AMF. Strategies are required to modulate the safety and efficacy of AMF to improve the therapeutic effect (Xiao *et al.*, 2016).

Hollow structured porous nanospheres integrating hollow interior with porous shell into one nanostructure are attracted more research attentions because of their unique properties, as good permeability, high surface area, low density as well as excellent loading capacity (Nadia and Mehrez, 2018). The void in the hollow structures could provide space for loading of guest molecules or particles, making them attractive in drug delivery. Ultrasonication has been used for the preparation of hollow structured porous compounds. Regarding the preparation of porous silica nanoparticles, it aims at creating free radicals that facilitate the formation of distributed and well stabilized nanoparticles (Jihan *et al.*, 2015).

Strong shearing action on oil-in-water droplet will produce long and thin structure that finally breaks up to nano droplets. Hereby, the current research was designed to prepare oil/water (O/W) nanoemulsion of compartmentalized hollow silica nanospheres by ultrasonication of an oil-water-surfactant system in the presence of silica source (tetraethyl orthosilicate; TEOS) together with a common cationic surfactant and oil nominated cetyltrimethylammonium bromide (CTAB) and castor oil, respectively.

The current work was conducted to prepare compartmentalized hollow silica nanospheres encapsulated with amifostine (AMF) as a model for drug delivery which investigate the protective role of the amifostine nanoemulsion drug model against increasing podocyte mRNA expression and progressive podocyturia in nephrotoxicity.

MATERIALS AND METHODS

Cisplatin and amifostine were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Ultrapure deionized water was generated using a Millipore Milli-Q plus system. Male Wister rats, weighing 180-200 g, were purchased from the animal house of the National Research Centre (Cairo, Egypt). Animals were maintained under standard conditions of ventilation, temperature (25±2°C), and light/dark cycle (12/12 h). The rats were housed in clean polypropylene cages and provided with free access to water and standard diet. *In vivo* experiments were carried out in accordance with guidelines and protocols approved by the Ethics Committee of the National Research Centre (Cairo, Egypt), animal study was ethically approved with ethical number (17/118).

After 10 days of acclimatization period, 50 rats were randomly and equally divided into five equal groups: gp1 NC i.p injected with saline daily for one month, gp2 (SiNPs) i.p. injected with particles of silica nanoemulsion (SiNPs) in a dose of 150 mg/kg/day for one month, gp3 (Cis) i.p. injected twice with cisplatin in a dose of 20 mg/kg body weight to induce nephrotoxicity according to Dósa *et al.* (2017), gp4 (SiNPs@AMF) i.p. injected with particles of silica nanoemulsion of amifostine in a dose of 150 mg/kg/day for one month and gp5 (SiNPs@AMF-Cis) i.p. injected with particles of silica nanoemulsion of amifostine (SiNPs@AMF) in dose of 150 mg/kg/day for three weeks then injected with two doses of cisplatin (20 mg/kg) followed by amifostine nanoemulsion i.p. injection to complete one month.

0.66 g of CTAB was dissolved in 12.2 ml of deionized water at room temperature. Afterward, an oil phase solution containing 1 ml of TEOS and 0.13 ml of castor oil was added to the aforesaid aqueous solution with stirring at 1000 rpm for 5 min to generate a simple oil-in-water [O/W] emulsion system. The reaction mixture was then sonicated using an ultrasonic bath [Branson 2510R-DTH Ultrasonic Cleaner, 100 W, 42 kHz], supplemented with mechanical stirring at 1000 rpm [IKA, EuroST D S1] for 15 min at room temperature. After that, a cloudy mixture was obtained and then left to stand for another 24 h. The nanoemulsion particles of silica [SiNPs] were isolated by centrifugation at 15000 rpm for 30 min. The product obtained was further washed in sequence with ethanol and deionized water to remove unreacted chemicals. To encapsulate the drug into the formed silica nanoemulsion 150 mg of Amifostine was added to the oil part of TEOS

and castor oil and then added to aqueous phase of CTAB solution.

At the end of experiments, animals were kept fasting for 12 h before blood sampling. Blood was withdrawn from the retro-orbital venous plexus of the eye using a heparenised capillary tube, under light anesthesia by diethyl ether. Blood was collected in tubes for biochemical analysis of TIMP-1, osteopontin, RANTS and homocysteine. Kidneys were removed quickly, rinsed and perfused with ice cold saline to remove blood cells. The kidney tissue was collected on TRIzol reagent for determination of gene expression levels.

TIMP-1, osteopontin and RANTS levels were estimated in serum using sandwich enzyme-linked immunosorbent assay (ELISA) kits purchased from Bioneovan Company (Q935+MW7, Keyuan Rd, Daxing District, Beijing, China). HPLC was used for the assessment of serum homocysteine according to Hussein *et al.* (2020). Relative expression of podocin and nephrin mRNA were analysed in kidney tissues using quantitative Real Time PCR.

400 µl serum was mixed with 30 µl of 1.2mol/l trichloroacetic acid (TCA), mixed well and incubated in ice for 30 min to precipitate protein. After centrifugation for 20 min at 3200 rpm and 4°C, supernatants were filtered through a hydrophilic 0.45-µm polyvinylidene fluoride (PVDF) membrane filter. HPLC condition: 50 µl from the filtered supernatant was injected into HPLC; separation was achieved on RP column (C18 × 25 × 0.46 cm × 5 µm). The mobile phase consisted of sodium phosphate monobasic monohydrate (40 mmol/l), heptanessulfonic acid (8 mmol/l) and 18% methanol (v/v). pH was adjusted to 3 by adding drops of phosphoric acid; the mobile phase was then filtered through a membrane filter (0.45 µm) and eluted at a flow rate 1 ml/min at 40°C using a UV detector that was set at 260 nm.

TRIzol extraction chemical (Invitrogen, Germany) was used to isolate the total genomic RNA of kidney tissues of all treated animals. After completion of the isolation procedures, RNA pellet was stored in DEPC-treated water. To digest the potential DNA residues, pellet of the isolated RNA was treated with RNAse-free DNAse kit (Invitrogen, Germany). Purity of total RNA was assessed spectrophotometrically by the ratio of A_{260}/A_{280}nm. RNA aliquots were utilized immediately for reverse transcription. First Strand cDNA Synthesis Kit (RevertAid, MBI Fermentas®, Germany) was used to synthesize the cDNA copy from the isolated RNAs via reverse transcription reaction (RT). RT reaction program of 25°C for 10 min, one hour at 42°C, and 5 min at 95°C was used to obtain the cDNA copy of kidney genome. Finally, tubes of reaction containing cDNA copies were collected on ice up to use for cDNA amplification.

SYBR Premix Ex TaqTM kit (TaKaRa, Biotech. Co. Ltd.) was used to perform the qRT-PCR analyses using the synthesized cDNA copies from kidney tissues. PCR reactions were set up in 25 µl reaction mixtures containing 12.5 µl 1× SYBR Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µl 0.2 µM sense primers, 0.5 µl 0.2 µM antisense primer, 6.5 µl distilled water, and 5 µl of cDNA template. The reaction program was allocated to three steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to three steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. For each reaction, a melting curve profile was conducted**.** The quantitative values of the target genes were normalized on the expression of the housekeeping gene. The $2^{-\Delta\Delta CT}$ method was used to determine the quantitative values of the specific genes to the ß-actin gene (Patankar *et al.*, 2016).

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. All data expressed as mean±standard error. Data were analyzed using SPSS (Version 16). Significant difference was considered at the level of $P < 0.05$.

RESULTS AND DISCUSSION

Induction of nephrotoxicity in experimental animals Cis group caused the TIMP-1, Osteopontin to be significantly elevated in comparison with NC group. Moreover, nephrotoxic animals recorded a significant increase in RANTS. Meanwhile, nephrotoxic animals protected with SiNPs@AMF recorded a less observable increase in serum TIMP-1, osteopontin as well as RANTS compared to NC animals indicating a better improvement. On the other hand, no significant difference in

the level of these parameters was observed between rats treated with either silica or SiNPs@AMF in comparison with NC animals (Table 1).

No significant difference in the level of homocysteine was observed between rats treated with either silica or SiNPs@AMF and normal control animals. On the other hand, induction of nephrotoxicity in experimental animals Cis group caused a significant increase in homocysteine. Meanwhile, nephrotoxic animals protected with SiNPs@AMF recorded a less observable increase compared to NC animals (Table 1).

Nephrotoxic animals recorded a significant decrease in expression of Podocin mRNA and Nephrin mRNA that is strongly down-regulated in Cis administered group. Meanwhile, nephrotoxic animals protected with amifostine nanoemulsion recorded a less significant decrease in expression of Podocin and Nephrin genes compared to NC animals. On the other hand, no significant difference in the level of these parameters was observed between rats treated with either silica or SiNPs@AMF and NC animals (Table 2).

Nanoemulsion based on silica nanoparticles are chosen for encapsulation the model drug; (AMF) for many reasons, which are the silica nanoemulsion can be used for carrying bioactive molecules and protect them from degradation can shield them from degradation under the effect of physiological conditions. On the other side, allow the drug to release in controlled state. Extend their blood movement, enhance disease targeting and minimize side effects to healthful tissues. The ultra-sonication technique was selected due to its capability to disperse the formed nanoemulsion and kept these particles in dispersing state without noticeable aggregation. In addition, there was no need to use an extra chemical such as acids or base to accelerate and catalyze the hydrolysis of TEOS.

Table 2. Relative expression of Podocin and Nephrin mRNA levels in different studied groups

Groups	Parameters	
	Nephrin mRNA	Podocin mRNA
NC	1.74±0.012[a]	2.1±0.03[a]
SiNPs	1.8±0.004[a]	2.2±0.008[a]
Cis	0.9±0.002[b]	0.76±0.004[b]
SiNPs@AMF	1.87±0.13[a]	2.0±0.015[a]
SiNPs@AMF- Cis	1.56±0.007[c]	1.8±0.006[c]

Values are represented as means±SE. Similar superscripts within same column indicate non-significant, while different superscripts within same column indicate significant at P<0.05 (one-way ANOVA).

Nephrotoxicity induced by drug was very common and from all Cis nephrotoxicity led to massive health and economical worldwide (Beigrezaei and Nasri, 2017). The mechanism of cisplatin as anticancer included the formation of highly reactive platinum complexes that bound with nucleophilic DNA through intra-strand and inter- strand crosslinking by guanine nucleotide. These events resulted in denaturation of DNA and thus cell cycle arrest. However, the mechanism of cisplatin nephrotoxicity differed from its activity as anticancer (Qi *et al.*, 2019). Cisplatin through cytochrome P450 (CYP) in microsome and mitochondrial dysfunction resulted in the formation of reactive oxygen species (ROS) and the renal tissue damage. This resulted in acute renal failure by induction of oxidative damage, inflammatory

Table 1. Effect of (SiNPs@AMF)on(TIMP-1), osteopontin and RANTS levels in cisplatin nephrotoxicity

Groups	Parameters			
	TIMP-1 (Pg/ml)	Osteopontin (Pg/ml)	RANTS (Pg/ml)	Homocysteine (µmol/l)
NC	17.9±3.7[a]	33.3±1.2[a]	469.7±14.2[a]	4.7±0.08[a]
SiNPs	21.4±4.2[a]	36.8±1.07[a]	507.8±4.4[a]	5.1±0.04[a]
Cis	137.2±16.8[b]	63.5±0.8[b]	884.8±13.3[a]	10.1±0.3[b]
SiNPs@AMF	26.2±3.09[a]	35.2±1.3[a]	501.9±7.19[a]	5.2±0.08[a]
SiNPs@AMF-Cis	61.7±5.4[c]	52.3±2.1[c]	658.3±20.4[c]	5.92±0.12[c]

Values are represented as means±SE. Similar superscripts within same column indicate non-significant, while different superscripts within same column indicate significant at P<0.05 (one-way ANOVA).

cytokines, tubule-interstitial inflammation as well as apoptosis/necrosis of renal tubular cells (Saisyo *et al.*, 2016). Different adjuvants as antioxidants, modulators of nitric oxide and anti-apoptotic agents were studied for their beneficial effects on cisplatin-induced renal injury (Chtourou *et al.*, 2016).

In the present study, the ameliorating potentialty of SiNPs@AMF against cisplatin-induced nephrotoxicity in rats was demonstrated. Injection of cisplatin (20 mg/kg, i.p.) to the rats led to noticeable nephrotoxicity in Cis group compared to control group that was in agreement with previous reports**.** Cisplatin induced nephrotoxicity was further evidenced from histopathological studies. Kidney specimens from rats treated with SiNPs@AMF and Cis showed moderated reduction in Cis-induced pathological alterations as mentioned in previous study (Nadia and Mehrez, 2018).

Patients with chronic kidney disease had many abnormalities in protein and amino acid metabolism. One of these variations involved an increased plasma concentration of the sulphur-containing amino acid homocysteine (Workeneh and Mitch, 2022). Elevated homocysteine level had been found among people with poor renal function, such as end stage renal disease (Workeneh and Mitch, 2022), and many studies had linked higher homocysteine levels to a greater decline of glomlular filtration rate **(**Huang *et al.*, 2019). These findings were established with our results where homocysteine level was elevated in rats injected with Cis comparing with NC group. As renal failure caused elevated serum homocysteine level, the relationship may also be with other factors by different mechanisms: (i) disturbance of homocysteine disposal in the kidneys themselves and (ii) impairment of extrarenal homocysteine metabolism (Huang *et al.*, 2019). It was found that homocysteine caused endoplamic reticulum (ER) stress in proximal tubular cells. Homocysteine was more sensitive to cisplatin-induced acute kidney injury by enhancing ER stress.

The pretreatment with SiNPs@AMF in the study was also able to attenuate the significantly detected increase of TIMP1 of Cis-protected rats where the most effective one was SiNPs@AMF, indicating the potential effect of this nano model drug against kidney damage (Perše and Veceric-Haler, 2018). The activation of TIMP-1 was responsible for the deposition of extra cellular matrix following the kidney damage (Maraoka *et al.*, 2019). TIMP-1 was the first-discovered natural collagenase inhibitor and exhibited diverse biological functions.

Osteopontin was a pleiotropic cytokine that is broadly expressed and upregulated during inflammation. Secreted osteopontin could bind to αVβ3 integr in (vitronectin receptor) and could induce phosphoinositide-3-kinase/Akt dependent NF-κB activation (Mushtaq *et al.*, 2021). Osteopontin could also induce NF-κB activation through both IKK- and extracellular signal-regulated kinase-mediated pathways, which stimulated uPA-dependent matrix metalloproteinase 9 (MMP-9) activation. In addition, previous studies by Kaleta (2019) showed that αVβ3 integrin (one of the osteopontin receptors) was a critical regulator of proteinuria. Our results also suggested that increasing in osteopontin level was a good indicator of inflammation in Cis-induced toxicity group. In the current study, the protection with SiNPs@AMF had a significant decreases in osteopontin level indicating the protective efficiency of SiNPs@AMF.

The ultrafiltration of plasma components through the formation of primary urine in the glomerulus was one of the pivotal functions of the kidney. The glomerular filtration barrier was formed of three layers: the glomerular basement membrane (GBM), the endothelium and the podocyte. The podocyte foot processes were joined just above the GBM with a special structure known as the slit diaphragm. This structure bridged the filtration pores between adjacent foot processes. According to experimental animal models and some human data. However, many studies deduced the direct role of the slit diaphragm in the pathogenesis of proteinuria. Their findings suggested that the slitdiaphragm was pivotal for the maintenance of the glomerular filter. Nephrin and podocin were the main protein in the podocyte slit diaphragm where it had an important role in maintaining the glomerular filter and the remarkable down-regulation of nephrin mRNA expression (Wang *et al.*, 2019).

Authors deduced that podocin and nephrin expression was significantly decreased in these patients; the expression of podocin and nephrin genes in biopsies from patients with

primary acquired nephrotic syndrome. Our results are in agreement with their investigations: podocin expression was reduced in Cis group. Two mechanisms were suggested for the down regulation of podocin and nephrin mRNA in Cis group. One was the characteristic structural alteration of glomerular epithelial cells or alteration of foot processes and filtration slits. The other was loss of podocytes (Perico *et al.*, 2016).

CONCLUSION

Nephrotoxicity induced by drugs as cisplatin recording elevation in the level TIMP-1, osteopontin, RANTS. Treatment with (SiNPs@AMF) attenuated the levels of TIMP-1, osteopontin, RANTS compared to the control group. Down regulation of expression of podocin mRNA and nephrin mRNA gene in cis group was noted. Contrarily, these disturbances were augmented by protection of (SiNPs@AMF) injection. The obtained results were considered as potential implications that offered a new approach in attenuating drug induced nephrotoxicity.

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