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Study of Urinary Biomarkers for Nephrotoxicity in Wistar Rats

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ABSTRACT

Nephrotoxicity is the second most persistent cause of drug withdrawal. In this study, we have determined the presence of nephrotoxic biomarkers in experimentally-induced site specific kidney injury. This study include measurement urinary clusterin, microalbumin, traditional serum nephrotoxic biomarkers (creatinine, urea, albumin, total protein), qualititative urine analysis and histopathological examination of kidney in rat models treated with carboplatin and ibuprofen-induced renal tubular injury. Female wistar rats were divided into four groups of six animals each were treated as follows: (1) Normal saline (0 mg kg^{-1} intraperitoneally) (2) Carboplatin (250 mg kg^{-1}, intraperitoneally) (3) 0.5% Carboxy methyl cellulose (0 mg kg^{-1}, orally) (4) Ibuprofen (800 mg kg^{-1}, orally). Blood urea levels increased significantly in response to carboplatin and ibuprofen treated rats indicating potential nephrotoxicity. In carboplatin and ibuprofen treated rats, urinary clusterin was dramatically increased indicating dysfunction of proximal tubule. Additionally carboplatin and ibuprofen treated rats showed proteinuria, microalbuminuria and hypoalbuminemia revealing dysfunction of either distal tubule or loop of henle. The data suggested potential proximal tubular damage alongwith damage to distal tubule and loop of henle in presence of carboplatin and ibuprofen. Therefore, combinatorial measurement of clusterin and microalbumin with other nephrotoxic biomarkers such as kidney injury molecule-1, Cystatin C, Beta-2 microalbumin and trefoil factor 3 might work as powerful tool for highly effective screening of nephrotoxicity.

Key words: Urinary biomarkers, glomerular injury, renal tubular injury, carboplatin, ibuprofen

INTRODUCTION

Nephrotoxicity is the second most persistent cause of drug withdrawal. This challenging problem still persists; causing many drugs to fail in late in their development process. Therefore, it is important to detect nephrotoxicity at early stage of drug development. This is due to traditional kidney function test such as serum creatinine and serum urea. This parameters have proven to be insensitive and non-specific, displaying changes only after significant kidney injury has occurred. Thus, there is a need for better early biomarkers of acute kidney injury (Maddali, 2009). Recently, the Food and Drug Administration and the European Medicine Agency (EMEA) published a report concerning the qualification of seven urinary nephrotoxic biomarkers, including total protein, albumin, kidney injury molecule-1, clusterin, β_2-microglobulin, cystatin C and trefoil factor 3, for particular uses in regulatory decision-making (Tonomura *et al*., 2010). Moreover, Microalbumin (Low Molecular Weight Protein) has been recently recognized as a novel nephrotoxic biomarker

(Vaidya *et al*., 2008). However, available information concerning comprehensive measurements and a comparison of the usefulness of these biomarkers for early detection of nephrotoxicity is limited. In the previous study, Gentamicin is reported to induce glomerulus and tubular damage in rats but still not evaluated site-specific nephrotoxicity (Lakshmi and Sudhakar, 2010). Renal tubular damage is well-known after treatment with anti-cancer drugs. Cisplatin is widely used as anti-cancer agent against solid tumors of testes, ovaries etc. Cisplatin-induced renal tubular injury by generation of reactive oxygen species and lipid peroxidation but still exact mechanism of nephrotoxicity by cisplatin is not fully elucidated (Abdin *et al*., 2008). Similarly, Carboplatin is a second-generation platinum-containing anti-cancer drug is currently being used in the clinic against lung, ovarian, head and neck cancers. It was developed in an attempt to overcome side-effects such as renal damage, peripheral neuropathy and ototoxicity have been found with its parent compound cisplatin (English *et al*., 1999). Glomerular impairment is generally absent or mild with small reduction in GFR as well as rare cases of Acute Renal Failure (ARF) have been described in adults treated with carboplatin. ARF and occasionally Chronic Renal Failure (CRF) have been reported in children treated with high-dose of carboplatin. Chronic subclinical tubular damage manifested by increased urinary retinol binding protein excretion is reported in carboplatin treated patient population (Skinner, 2010). The outstanding effects of the PGs include their cytoprotective properties in the gastrointestinal tract and control renal function in the kidney (Al-Turki *et al*., 2010). NSAIDs nonspecifically inhibit cyclooxygenase, an enzyme involved in renal prostaglandin synthesis and this inhibition is believed to promote compensatory vasodilatory disequilibrium, leading to a deterioration of glomerular filtration rate. Ibuprofen treated rats' revealed cortical and medullary congestion and microscopically acute tubular necrosis, moderate inflammation and interstitial congestion (Kumar *et al*., 2010). Therefore, objective of this study was to examine usefulness of urinary clusterin and microalbumin in a carboplatin and Ibuprofen-induced nephrotoxicity as well as to identify damaging part of the nephron after carboplatin and ibuprofen treatments.

MATERIALS AND METHODS

Chemicals: Carboplatin and Ibuprofen were obtained from Cadila Health Care Limited. All other chemicals used in this study were of the highest purity available.

Animals and housing condition: About 6-8 weeks old female wistar rats were obtained from Animal Research Faculty of Zydus Research Centre. Experiments were complied with the CPCSEA Guidelines of this institution. All animals were allowed free access to food (Standard Chakan brand pellet diet) and water (Clean water purified by Aqua guard water system) except for when urine was being collected. The animals were housed under 12 h light/dark (Lights on 8:00-20:00) in individual ventilated cage system. The temperature condition of animal housing was 22±3°C.

Experimental design: The experiment design is shown in Table 1. The animals were allocated to four groups of six animals each: a normal saline-treated group, carboplatin-treated group, 0.5% CMC-treated group, Ibuprofen treated group. Carboplatin was dissolved in saline solution at the required concentration just prior to use. Ibuprofen was dissolved in 0.5% CMC solution at the required concentration just prior to use. Repeated dose of 250 mg carboplatin per kg of animal body weight was administered intraperitonially while repeated dose of 800 mg ibuprofen per kg of body weight was administered orally based on the weight of animal (5 mL kg^{-1}). All the animals were acclimatized to experimental room condition for 5 days. All animal were sacrificed on day 5.

Table 1: Experimental design

Group	Treatment groups	Duration (days)	Dosage (mg kg^{-1})	No. of rats/group	Route of administration
I	Normal saline	4	0	6	Intraperitoneally
II	Carboplatin	4	250	6	Intraperitoneally
III	0.5% CMC	4	0	6	Orally
IV	Ibuprofen	4	800	6	Orally

Measurement of urinary biomarkers: Urinary clusterin was performed using the Rat Clusterin Elisa Kit (Life Diagnostic Inc., Cat. No. 3300-2).Urinary microalbumin was performed using the Rat Microalbumin Elisa Kit (Kamiya Biomedical Company, Cat. No. KT-354).

Urine analysis: Animals were kept in metabolic cages on day 4 for the collection of urine sample after taking body weights. The animals were given measured volume (100 mL) of distilled water and fasted overnight in metabolic cages. After measuring urine volumes, urine samples centrifuged at 1000 RPM for 10 min with cooling for analysis. Urine samples from all the animals were analyzed for total protein, blood cells and leucocytes by automatic urine analyzer (Clinitek-status).

Blood chemistry: Blood samples were collected from retro-orbital plexus of rat in 1.0 mL eppendorf having EDTA (0.5 mL blood) for hematology and in plain eppendorf (1 mL blood) for separation of serum. Blood samples for serum biochemical analysis were collected from retro-orbital plexus of rat in plain eppendorf. The blood was allowed to clot and then centrifuged at 4000 rpm for 5 min at ambient temperature. Serum albumin, total protein, creatinine, urea levels were analyzed using automatic biochemical analyzer (Daytona).

Pathological examination: After blood collection all the animals were sacrificed on day 5. Kidney was collected and weighed. Kidney was preserved in 10% formaline. Then kidney was processed and slides were prepared for histopathological examination.

Mortality: All the animals were observed daily till day 5 of the experiment for any abnormal physical or behavioral changes and mortality throughout the dosing period.

Statistical analysis: Statistical analysis was performed using Graph Pad Prism Version 4.00. Data were analyzed for dose wise comparison. ANOVA (Analysis of Variance) was used for the comparison of different dosage groups with the control group for different parameters. Comparison of dosage groups with the control group was done on the basis of individual group data. Post hoc test to analyze data after ANOVA was done using Dunnett's test (parametric) or Dunn's test (non parametric). Tests used in analysis of data were done at 5% level of significance.

RESULTS

Effect of carboplatin and ibuprofen on urinary clusterin and microalbumin: As shown in Fig. 1a and b, after administration of carboplatin or ibuprofen, rats showed significant ($p<0.01$) increase in microalbumin and clusterin level on day 5 when compared with the respective vehicle treated rats.

Effect of carboplatin and ibuprofen on qualitative urine analysis: As shown in Table 2, carboplatin or ibuprofen treated rats showed significant protenuria on day 5 when compared with

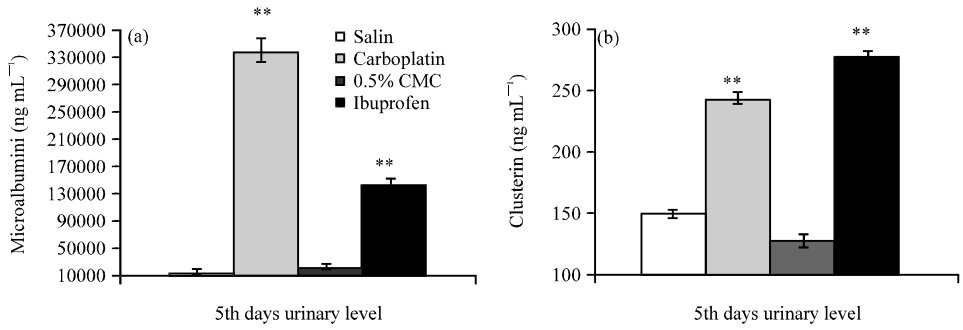

Fig. 1 (a-b): Urinary biomarkers in nephrotoxic rat models. The urinary level of (a) microalbumin and (b) clusterin after treatment with carboplatin (250 mg kg^{-1}) or Ibuprofen (800 mg kg^{-1}) or respective vehicle control. Data are expressed as Mean±SEM. The statistical significance of differences between treated and control groups was determined using ANOVA followed by Dunnett's (parametric) or Dunn's test (nonparametric). *p<0.05 significant compared with control, **p<0.01 significant compared with control

Table 2: Qualitative urine analysis in the normal saline, carboplatin, 0.5% CMC and ibuprofen groups

Treatment group	Urinary protein (mg dL^{-1})	Urine output (mL)	Urine leucocytes	Urine blood
Saline	Nil	18.8	Nil	Absent
Carboplatin	300	2.0	Trace	+++
0.5% CMC	Nil	14.7	Nil	Absent
Ibuprofen	100	5.8	Nil	Absent

the respective placebo. However, the amount of urine protein excretion (300 mg dL^{-1}) was very high with carboplatin as compared to that ibuprofen treated rats (100 mg dL^{-1}). Simultaneously, they showed significant decrease in urine output indicating marked decrease in glomerulus filtration rate. In addition to this, carboplatin treatment also showed presence of leucocytes and blood cells in urine.

Effect of carboplatin and ibuprofen on blood chemistry: Carboplatin or ibuprofen treated rats showed significant decrease in serum proteins such as albumin and total proteins when compared with their respective control groups (Fig. 2a-d). Besides, significant rise was observed in serum urea levels with no remarkable changes in serum creatinine levels.

Effect of carboplatin and ibuprofen on kidney pathology: Histopathological changes in kidney following carboplatin and ibuprofen treatment are shown in Fig. 3 and Table 3. There were renal tubular changes observed viz. minimal to severe dilatation of tubules, minimal to mild diffuse mineralization in tubules in paracortical region and minimal to mild degeneration of tubules with no alteration in the glomeruli (Fig. 3). Additionally carboplatin treated animals also showed presence of hyaline casts in tubular lumen region.

Percentage mortality after carboplatin and ibuprofen treatment: As shown in Table 4, carboplatin and ibuprofen treated rats showed 33.3% mortality due to severe renal toxicity.

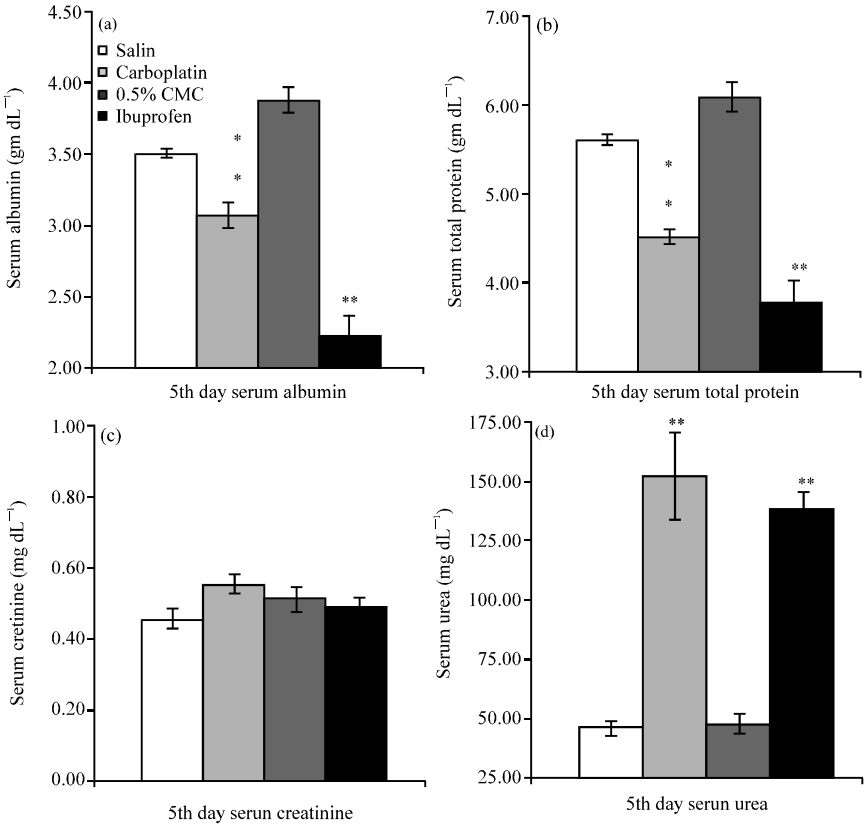

Fig. 2 (a-d): Serum chemistry in nephrotoxic rat models. (a) The serum levels of albumin, (b) total protein, (c) creatinine and (d) urea after treatment with carboplatin (250 mg kg^{-1}) or ibuprofen (800 mg kg^{-1}) or respective control groups. Data are expressed as Mean±SEM. The statistical significance of differences between treated and control groups was determined using ANOVA followed by dunnett's(parametric) or dunn's test(nonparametric). *$p<0.05$ significant compared with control, ** $p<0.01$ significant compared with control

Table 3: Histopathological changes in the kidney after carboplatin or ibuprofen administration

	Renal injury									
			Tubular region							
	Glomerulus		Dilatation of tubules				Degeneration of tubules			
Treatment	-	+	-	++	+++	++++	-	++	+++	++++
Saline (0 mg kg^{-1})	6	0	6	0	0	0	6	0	0	0
Carboplatin (250 mg kg^{-1})	6	0	0	1	2	1	6	0	0	0
0.5% CMC (0 mg kg^{-1})	6	0	6	0	0	0	6	0	0	0
Ibuprofen (800 mg kg^{-1})	6	0	0	4	0	0	0	2	2	0

The numericals represent the number of rats affected (n = 6/group). The histological changes were arbitrarily scored as follows: -: No abnormal findings, +: Minimal, ++: Moderate, +++: Marked, ++++: severe

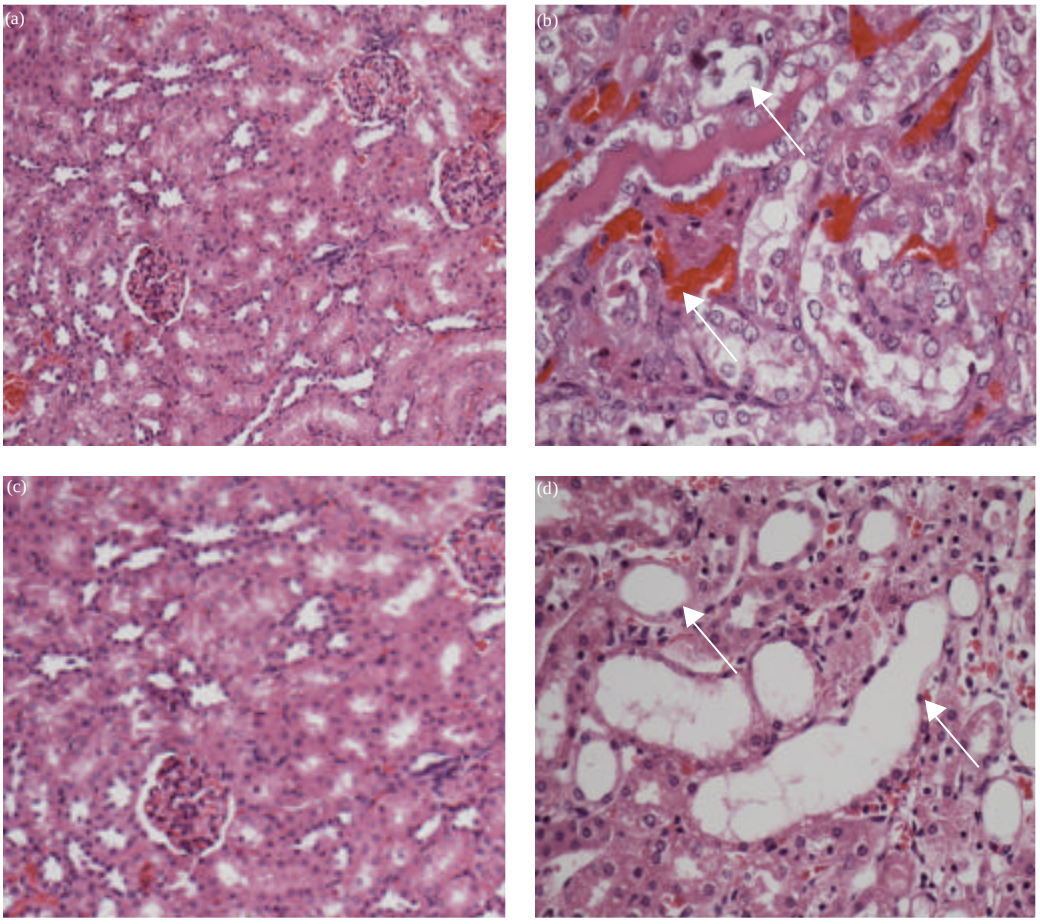

Fig. 3 (a-d): Effect of carboplatin (250 mg kg^{-1}) and ibuprofen (800 mg kg^{-})[1]treatment on rat kidney. Histopathological examination of rat kidney tissue in (a) normal saline (G), (b) carboplatin (H), (c) 0.5% CMC (I) and (d) ibuprofen (J) groups by high-power (40X) microscopy. Rats were treated with a repeated dose of normal saline (0 mg kg^{-1}), carboplatin (250 mg kg^{-1}), 0.5% CMC (0 mg kg^{-1}) and ibuprofen (800 mg kg^{-1}). Aerrowheads indicate dilatation of tubules and tubular necrosis

Table 4: Percentage mortality in the saline, carboplatin, 0.5% cmc group and ibuprofen groups

Treatment	Dose (mg kg^{-1})	Mortality (%)
Saline	0	0.0
Carboplatin	250	33.3
0.5% CMC	0	0.0
Ibuprofen	800	33.3

DISCUSSION

In the present study, we evaluated usefulness of urinary clusterin and microalbumin as a biomarkers of nephrotoxicity as well as to identify damaging part of the nephron after carboplatin

and ibuprofen treatment. We also calculated the accuracy and sensitivity of detection of urinary clusterin and microalbumin in renal tubular injury model induced by carboplatin or Ibuprofen. Cisplatin caused structural alteration in Proximal Convovulated Tubule (PCT) and Distal Convovulated Tubule (DCT) part of the nephron after 2 weeks treatment (Abdelmeguid *et al*., 2010). Carboplatin is a second generation platinum derivative which was developed to minimize side-effects such as renal damage, peripheral neuropathy and ototoxicity which were associated with its parent platinum compound cisplatin. There has been one report of acute renal failure following high-dose of carboplatin in a child (Frenkel *et al*., 1995). One case of acute renal failure and more than 50% reduction in GFR in six out of 39 patients receiving two courses of carboplatin and etoposide for relapsed neuroblastoma is reported by Frappaz *et al*. (1992). Chronic sub clinical renal tubular damage is also reported after carboplatin treatment (Goren *et al*., 1987). Previous study with carboplatin treatment revealed hypomagnesemia due to proximal tubular injury as well as glomerulus and distal tubule injury (Skinner, 2010). The previous study data showed that carboplatin in single high dose (256 mg kg^{-1}) caused renal dysfunction in rats as evident from levels of plasma creatinine, blood urea nitrogen and blood urea levels in time-dependent manner (Husain *et al*., 2004). In the present study, carboplatin and ibuprofen treated rats showed significant increase in serum urea levels that reflect the potential renal toxicity. Histopathological examination revealed significant toxicity at the proximal tubule in presence of carboplatin. Present study findings are parallel with the previous studies of carboplatin in rats.

It has been reported that clusterin is specific biomarker to proximal tubular damage (Dieterle *et al*., 2010). In contrast to clusterin, microalbumin (LMWP) can freely pass through glomerulus capillary wall due to their low-molecular weight and almost completely reabsorbed by epithelial cells of tubular part of the nephron (Tiwari, 2006). It has been reported that increased urinary excretion of low molecular weight proteins indicate proximal tubular toxicity and increased urinary excretion of high-molecular weight proteins such as albumin and total protein or decreased serum levels of this proteins indicate glomerular toxicity (Tiwari, 2006). In this study, carboplatin treatment showed an intense elevation of urinary clusterin and microalbumin as well as significant decrease in serum albumin and total protein. This may involve two mechanisms such as increased transglomerular passage of albumin due to loss of charge restriction and/or size restriction of glomerular capillary wall and an impairment of the reabsorption by the proximal tubule due to toxic injury (D'Amico and Bazzi, 2003). Previous report of cisplatin-induced nephrotoxicity rat model showed albiminuria which resulted from an inhibition of receptor-mediated endocytosis following inhibition of vacuolar H^+-ATPase in the renal proximal tubule indicated injury of proximal tubules but not to that of glomerulus (Takano *et al*., 2002). Carboplatin is second generation platinum compound of cisplatin. Therefore, presence of proteinuria and microalbuminuria in the carboplatin treated animals in this study, might due to proximal tubular injury and not the glomerular injury. This supports the hypothesis/speculation of proximal tubular toxicity by platinum derivatives such cisplatin and carboplatin.

In the published literature, NSAIDs are reported to cause renal injury by inhibition of prostaglandin synthesis results in decreased ability of the kidney to autoregulate the blood flow. Ketoprofen is reported to cause renal injury confirmed by increased serum levels of urea nitrogen, creatinine and histopathological change in the kidney (Safarchi *et al*., 2010). Ibuprofen is a non-steroidal anti-inflammatory drug which is chemically similar to the ketoprofen and prescribed as over-the-counter analgesic as well as in various somatic inflammatory conditions of musculoskeletal and dental origin. The nephrotoxicity of ibuprofen explained by their inhibition of prostaglandin synthesis and resulting interference with their physiologic action of prostaglandins in the kidney.

In one previous study, histopathological examination of kidney after 14 days treatment with ibuprofen (100 and 200 mg kg^{-1}) in rats showed acute tubular necrosis, moderate interstitial inflammation and degeneration of tubules which confirmed that ibuprofen cause renal tubular injury (Kumar *et al.*, 2010). In the present study, elevation of urinary clusterin might reflect proximal tubule damage. Additionally increased urinary excretion of microalbumin and decreased serum levels of albumin revealed tubular toxicity involving proximal tubule. Present histology reports also supported renal tubular injury in the ibuprofen 800 mg kg^{-1} group. Present study findings are parallel with the preclinical study report of Kumar *et al.* (2010) who showed renal tubular injury in rats treated with ibuprofen 100 and 200 mg kg^{-1} doses.

In addition to above, reduction in urine output in carboplatin group and ibuprofen group showed decline in glomerulus filtration rate. The decline in GFR may stimulate synthesis of vasodilatory prostaglandins that act in the reduction of afferent artiole resistance and thus reestablishment of renal plasma flow. The use of ibuprofen may inhibit this additional protective mechanism due to afferent arteriolar hypertonicity and absence of vasodilatory prostaglandins leading to reduction in renal plasma flow and reduction in GFR (Hosaka *et al.*, 2004). However, it is difficult at this stage to suggest this hypothesis for carboplatin.

In conclusion, urinary nephrotoxic biomarkers clusterin and microalbumin were measured in carboplatin and ibuprofen nephrotoxicity models and evaluated their usefulness as nephrotoxic biomarkers for site-specific early renal injury.

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