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Amelioration and Prognostic Significance of the Urine/Plasma Protein Creatinine Ratio Levels (uCr/pCr) as Predictive Biomarkers and its Change Patterns in Early Diagnosis of Cisplatin-Induced Nephrotoxicity of Solid Tumors

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ABSTRACT

Cisplatin (Cp) is extensively utilized as an antineoplastic drug employed in the treatment of miscellaneous solid tumors with serious side effects such as nephrotoxicity. Therefore, traditional biomarkers including urinary creatinine (uCr) and serum creatinine (sCr) are utilized for detection of Cp-induced renal injury. In this study, we compared changes in uCr/uCr gene expression levels (ml/min) at 1 ,2and 4 h and 2, 3, 4, 7,10,14 and 28 days after cisplatin infusion (0.75 mg/mL) versus baseline in 7 consecutive patients of solid kidney tumors with chronic kidney injury(CKI) and 3 consecutive controls without CKI. Furthermore, short-term cisplatin chemotherapy (STC, 1 day) was compared to long-term (LTC, 28days) treatment using plasma and urine creatinine and renal histology. We found CKI was associated with higher levels of sCr (149.5± 19.08versus 124 ± 23.54ng/mL, $P < 0.001$) and uCr (0.66± 0.11versus 0.94± 0.05, $P < 0.001$) compared with exposed controls. Patients with CKI associated LTC had increased level of sCr (79.1 ± 4.01versus 146.1 ± 6.66 ml/min,$P < 0.05$) and uCr (1.35 ± 0.08versus 57.66 ± 18.88 ml/min,$P < 0.05$) compared with CKI patients without cisplatin-treated tumors. Since patients with renal injury revealed significantly higher CKI scores and severe proximal tubular cells damage, significant differences were found for plasma or urine creatinine levels ratios. High expression of uCr/ sCr ratio in the renal is associated with nephrotoxicity in solid kidney tumors, suggesting uCr may play a role in proximal tubular injury of LTC, therefore, our data suggest that urinary uCr may be considered to be reliable markers to monitor renal injury in renal injury patients undergoing LTC.

Introduction

Cisplatin (Cp) is as a chemotherapeutic agent against solid tumors (Lebwohl & Canetta, 1988) and the anticancer effect of Cp is dose dependent, yet the risk of nephrotoxicity often precludes the use of higher doses to maximize the therapeutic effect. However, clinical use may be involved by its potential renal toxicity. A single injection of Cp may induce kidney epithelial cell injury, epithelial-to-mesenchymal transition (EMT), and progressive interstitial fibrosis, along with an upregulation of transforming growth factor-β1 (TGF-β1) (Yamate, Machida, Ide, Kuwamura, & Kotani, 2005; Yamate, Tatsumi, Nakatsuji, Kuwamura, & Kotani, 1995).

Despite years of research and hundreds of reports on cancer markers in oncology, the number of markers that have emerged as clinically useful is pitifully small (Bast et al., 2001; Hayes et al., 1996; Schilsky & Taube, 2002). Recent animal studies clearly indicated that the treatment of kidney injury (KI) should be started well before the rise of sCr and very early after the insult (Chiao et al., 1997; Conger & Falk, 1991; Kelly et al., 1996; Lieberthal, Sheridan, & Valeri, 1990). These studies proposed that more accurate and efficient measure for AKI diagnosis was urgently required (Slocum, Heung, & Pennathur, 2012). Lines of evidence showed that urinary NGAL, IL-18, Cys-C, KIM-1 and some other candidate molecules were believed as potential markers to diagnosis of AKI (Adiyanti & Loho, 2012; Edelstein, 2008). But until now, none of them are currently established well enough to replace serum creatinine as a marker of renal function. Sensitive biologic markers of renal tubular injury are needed to detect early KI because current KI diagnosing and staging criteria are entirely based on an increase in sCr or decrease in urine output. These tubular damage markers have been extensively investigated in the field of predicting the occurrence of acute kidney injury after various nephrotoxic insults, such as ischemia during cardiac surgery, sepsis, and administration of contrast medium (Bagshaw & Bellomo, 2007; Han, Wagener, Zhu, Wang, & Lee, 2009). In urinary biomarker studies, the diagnosis of KI, previously termed acute renal failure, is based typically on an elevation in the sCr and uCr concentrations. There is still no broadly accepted consensus on the degree of sCr and uCr elevations required to qualify for the diagnosis of KI. Consequently, factors influencing sCr could affect time to recognition of KI and lead to underestimating the severity of renal dysfunction over the course of KI. Aside from the well-recognized biological influences of age, muscle mass, catabolic rate and race (Jones et al., 1998; Levey, 1990).

Little research has been done in patients with chronic kidney disease. In this study, we aimed to investigate serum and urinary levels of the creatinine as proximal tubular damage marker in patients undergoing treatment with cisplatin of this marker to the severity of kidney injury as assessed by creatinine.

Materials and Methods

Animal Welfare

All experiments described in this study were performed in full accordance with the guidelines for animal experiments released by the National Institute of Animal Health. The protocol was approved by the Ethics Committee for Animal Clinical Research and all owners provided informed consent prior to enrollment into the study.

Animals and Cisplatin Administration

A total of 10 clinically healthy mature male (1-2 years old), mixed breed dogs (21-29 kg) were obtained from the Department of Clinical Sciences. The animals were acclimated to their

designated housing for at least 7 days before the first day of dosing. The dogs were assigned into 2 groups, control group (n = 3) and test group (n = 7) by a stratified randomization scheme designed to achieve similar group mean body weights. The animals were housed under an Institutional Animal Care and Use Committee–approved protocol and in separate stainless steel cages. The animals were provided commercial dog food twice a day and water ad libitum. Cisplatin (Mylan Pharmaceutical Co, Greece) was administered intravenously at 0.75 mg/kg/day for 5 consecutive days via cephalic vein and at an infusion rate of approximately 20 mL over 20 minutes.

Clinical Observation

Animals were checked twice daily (a.m. and p.m.) for mortality, abnormalities, and signs of pain or distress. Body weights were recorded twice before dosing on Day 1 and on the day of scheduled necropsies (Day 28). Food and water consumption were monitored twice daily, in morning and afternoon. Clinical signs including decreased food intake and water consumption, behavioral changes, motility, lethargy, ataxia, vocalization, unresponsiveness, and dehydration were recorded.

Biomarkers Analyses

Blood samples were collected via femoral vein and urine samples were collected via urethral catheter. Blood and urine samples were collected prior to dosing (Day 0) and after dosing on Day 1 (1 hr, 2 hr, and 4 hr post-dose) and Days 2, 3, 4, 7, 10, 14, and 28. The animals had access to drinking water and were fasted overnight prior to each scheduled sample collection. Blood was collected into tubes without anticoagulant and centrifuged within 1 hr of collection at 1,850 × g for 10 min at room temperature. The serum and urine samples were stored at – 80 ^{o}C until analysis. Serum and urine Cr and serum UN concentrations were measured using a chemistry auto analyzer (Prestige 24i analyzer, Boeki Medical Systems, Japan).

Histopathologic Examination

Each kidney was fixed in 10% neutral phosphate buffered formalin, processed in paraffin wax, sectioned (5 µm thickness), and stained with hematoxylin and eosin (H&E). Several consecutive paraffin sections were de-paraffinized three times with xylene for 7 min. After gradual rehydration in a series of graded alcohols concentrations and washing with deionized water, the sections were stained with H & E for 1 min, rinsed with deionized water and developed in tap water for 5 min. The tissue sections were destained by dipping the slide in acidified ethanol and rinsing in tap water. After washing with deionized water, the sections were stained with eosin for 30 s, dehydrated, and mounted. The histopathological findings were determined using light microscope.

Statistical Analysis

Data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp, Redmond, WA) for analysis. Using SPSS 20.0 statistical software (SPSS Inc, Chicago, IL), a repeated-measures ANOVA and paired-sample t test analysis were performed and differences were considered significant at values of $P<0.05$.

Results

Clinical Observations

For better evaluation of kidneys performance, clinical observation was carried out to compare with clinical signs due to cisplatin- induced toxicity which are summarized in Table 1 (number 1, 2, 3,

5, 6, 7 and 8 are test dogs and 4, 9 and 10 are control dogs). No deaths occurred in any of the treatment and control groups during the experimental period. A total of 10 consecutive dogs were included. Table 1 shows the clinical characteristics of all dogs enrolled in the study. Significant differences were observed between the two groups for the other baseline characteristics shown in Table 1.

Table 1
Clinical Observations of Patients Undergoing Chemotherapy During Treatment Periods

No	Decreased food consumption	Decreased drinking	Behavior and motility changes	Dehydration	Oral lesions	Vomiting	Mortality
1	+	+	+	+	+	+	_
2	+	+	_	_	_	+	_
3	+	+	_	+	_	_	_
4	_	_	_	_	_	_	_
5	+	+	_	_	_	+	_
6	+	+	_	+	_	_	_
7	+	+	+	+	_	+	_
8	+	+	+	+	+	+	_
9	_	_	_	_	_	_	_
10	_	_	_	_	_	_	_

Our results revealed that test group treated with cisplatin (0.75 mg/kg/day for 5 consecutive days) showed clinical signs of toxicity as indicated in Table 1, dogs number 4, 9 and 10 are control animals and dog number 1, 2, 3, 5, 6, 7 and 8 are test dogs which received CP. In this study because of low number of test animals and in order to control mortality, we performed some treatments to keep all the dogs alive during study course.

Histopathogical Examination

On histology, in the kidneys, there was marked interstitial necrosis, mononuclear inflammation and small numbers of neutrophils, vacuolation, and desquamation of epithelial cells in the renal tubules of the cisplatin-treated group. Cisplatin administration resulted in severe tubular damage especially proximal. Nevertheless, interstitial inflammatory reaction with lympho-plasmacytic infiltration, either diffuse or multifocal, was seen in the kidneys of all dogs. Infiltration of inflammatory cells was less pronounced in the medullary tissue, and infiltrating lymphocytes were often present in the chronic stage of CP. In addition, there were groups of tubules lined by highly vacuolated cuboidal epithelium. Furthermore, all dogs were dosed once with a high dose (HD) of Cp and renal histopathology was assessed. The examination revealed the expected changes associated with Cp-induced injury. The Cp-LD dosed animals indicated minimal tubular degeneration on day 1-5, on day 5 minimal to slight degeneration and on day 7 slight to moderate tubular necrosis was observed. More severe histopathological findings were observed in Cp-HD dosed rats. On days 1-7 we found minimal tubular necrosis and minimal to moderate tubular degeneration, on days 7-14 slight to moderate and on day 28 moderate to very severe tubular necrosis (Figure 1). Slight to severe inflammation were noticed in Cp-HD on day 28.

Control dogs exhibited no apparent histopathology changes in the kidney. CP produced epithelial cells injury of single-cell necrosis and tubular degeneration in segments of proximal tubules. Following treatment with CP, marked proximal tubule cell necrosis, tubular dilatation, vacuolization, and hyaline casts were observed. CP treatment in 2-week dogs produced more extensive damage in proximal tubules compared to control rats. In 4-week dogs, more severe damage was noted in the outer medulla and inner medulla compared to that in control dogs. The glomerulus did not appear to be affected by CP treatment in both age groups.

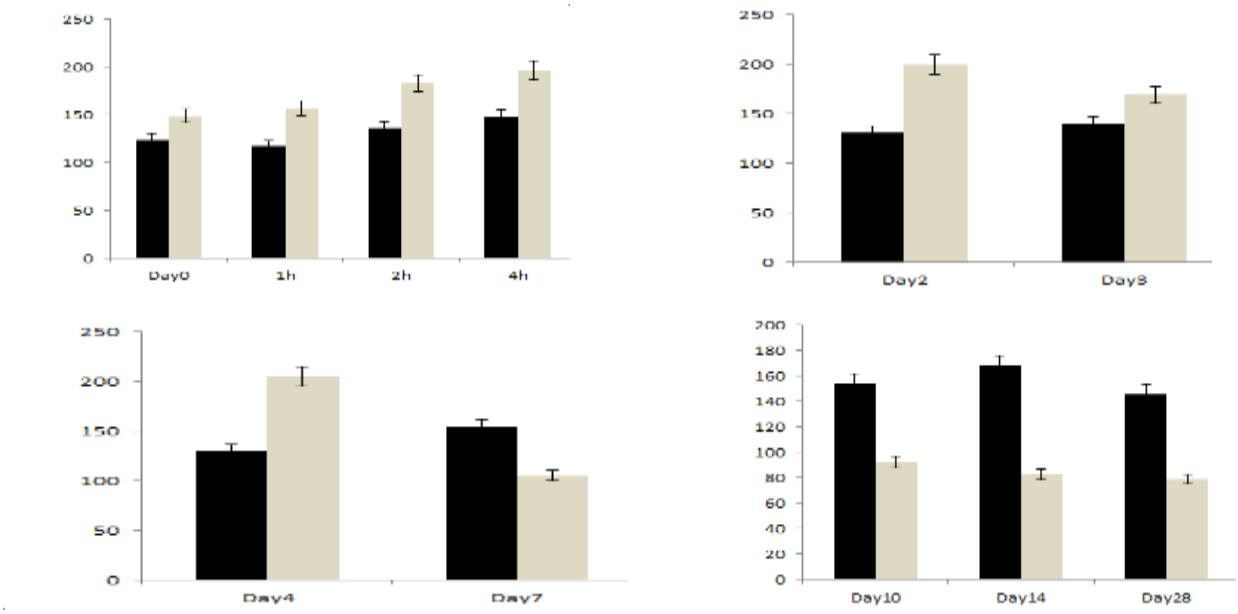

Figure 1. Changes in serum creatinine in all dogs at days 0,1,2,3 4, 7, 10, 14 and 28 after a single intravenous administration of cisplatin (0, 75mg/kg BW).

Statistical analysis was performed by ANOVA and t- test. Statistical significance is indicated by * $p<0.05$ compared to time matched control groups. Data are expressed as the mean ± S.E.M. (n = 10). Statistically significant changes are indicated by *$p < 0.05$ compared with control

Nephrotoxicity Biomarker in Kidney Tissue

Results for serum and urinary creatinine biomarker available on or measured with only one platform are summarized in Supplementary Tables 2 and 3.

Table 2

Comparison of the Mean Serum Creatinine Values of the Patients Whose Treatment Completed After Cisplatin Chemotherapy During Treatment Periods

T G	D0	1h	2h	4h	D2	D3	D4	D7	D10	D14	D28
sCr	157.9± 19.08	157.9± 21.77	183.07± 23.91	197.4± 41.69	200.2± 50.64	169.7± 28.28	205.7± 27.50	105.1± 13.48	92.5± 4.08	83.7± 10.58	79.1± 4.01
control	124± 23.54	117.6± 22.06	136.6± 24.22	148± 26.35	131.1± 30.10	140.5± 19.50	130± 18.23	154.1± 1.58	154.5± 8.22	168.6± 6.33	146.1± 6.66

Abbreviations: S; serum; D; day; T; time; G; group

Table 3

Comparison of the Mean Urinary Creatinine Values of the Patients Whose Treatment Completed After Cisplatin Chemotherapy During Treatment Periods

T G	D0	1h	2h	4h	D2	D3	D4	D7	D10	D14	D28
uCr	0.66± 0.11	0.78± 0.11	0.76± 0.11	0.95± 0.07	1.02± 0.08	0.88± 0.13	1.41± 0.20	1.80± 0.29	1.62± 0.35	1.45± 0.08	1.35± 0.08
control	0.94± 0.05	0.91± 0.06	0.91± 0.03	0.87± 0.02	0.87± 0.01	0.93± 0.008	0.79± 0.08	1.01± 0.10	0.99± 0.05	0.95± 0.02	1.003± 0.02

Abbreviations: U; urinary; D; day; T; time; G; group

In both groups, control displayed low levels of renal tissue creatinine marker expression. In CP-treated groups of both serum and urinary, expression levels of creatinine in the kidney tissue were significantly increased while expression levels of Cp were down-regulated in control groups. In 4 weeks of test groups, creatinine gene expression was significantly elevated but was unchanged in 4 weeks of control

groups both serum and urine. The expression levels of creatinine were changed in the kidney by CP in both serum and urine groups (Figures 1, 2 and 3).

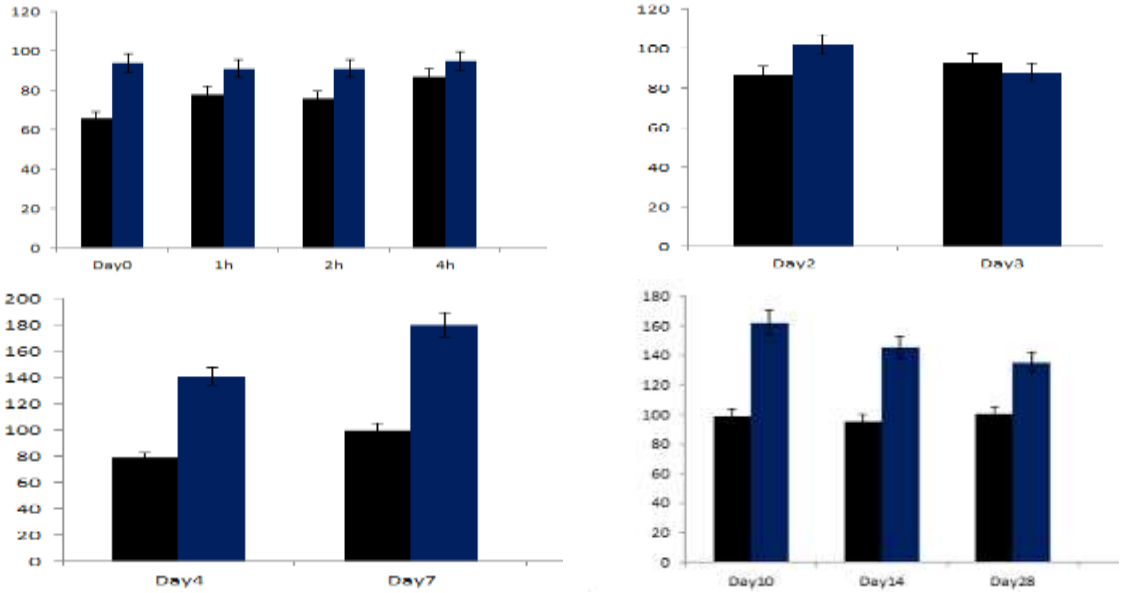

Figure 2. Changes in urinary creatinine in all dogs at days 0,1,2,3 4, 7, 10, 14 and 28 after a single intravenous administration of cisplatin (0, 75mg/kg BW).

Statistical analysis was performed by ANOVA and t- test. Statistical significance is indicated by * $p<0.05$ compared to time matched control groups. Data are expressed as the mean ± S.E.M. (n = 10). Statistically significant changes are indicated by *$p < 0.05$ compared with control.

There were no statistically significant linear correlations between baseline serum and urinary creatinine levels in the CKI groups. There was significant difference in the baseline serum and urinary creatinine concentrations between groups. The serum and urinary concentrations of creatinine increased in all dogs following Cp infusion despite the administration of large amounts of hydration. Compared to baseline, serum and urinary NGAL values were significantly increased at 28 days (Figures 1, 2 and 3).

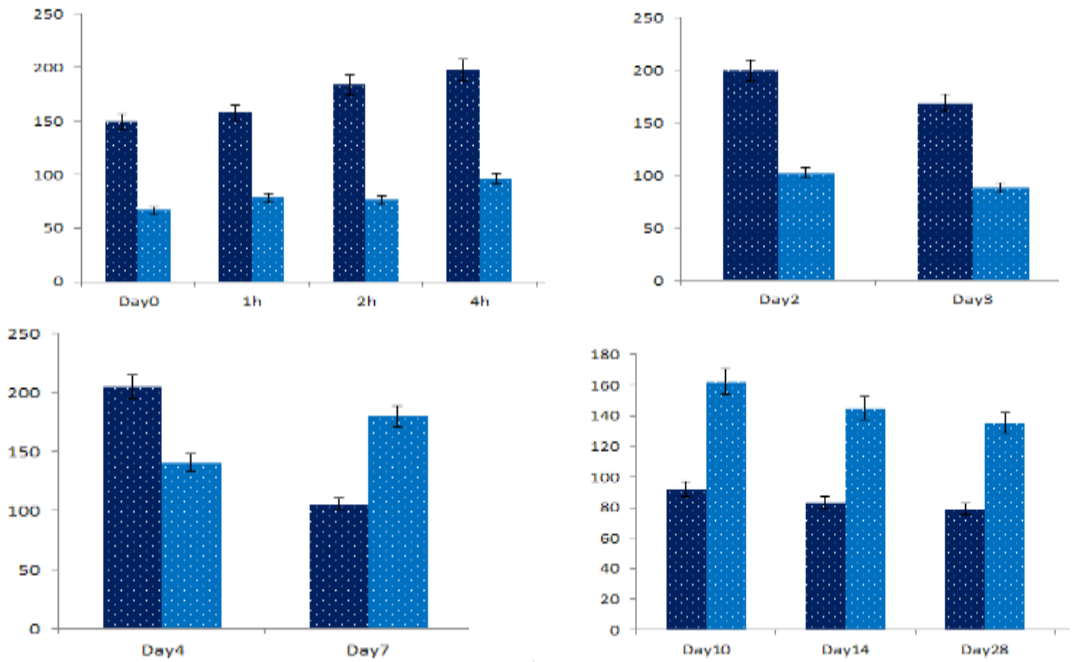

Figure 3. Changes in urinary creatinine in all dogs at days 0,1,2,3 4, 7, 10, 14 and 28 after a single intravenous administration of cisplatin (0, 75mg/kg BW).

Statistical analysis was performed by ANOVA and t- test. Statistical significance is indicated by * $p<0.05$ compared to time matched control groups. Data are expressed as the mean ± S.E.M. (n = 10). Statistically significant changes are indicated by *$p < 0.05$ compared with control

Discussion

As the know, renal are urodynamic organs and represent one of the main homeostasis body systems, and involved by multifarious varieties of chemical and medication materials (Abdel-Moneim & Said, 2007; Al Kahtani, Abdel-Moneim, & El-Sayed, 2014; Salem & Salem, 2011). So, should be noted that nephropathy and nephrotoxicity are the most significant side effects that may influence morbidity and mortality in patients. Medication-proved mentioned lesions are the fifth most usual agent of kidney injury (Haase & Mertens, 2010). Today, Crs and Crp biomarker levels and kidneys biopsy and specimens are utilized to early diagnosis these lesions (Haase & Mertens, 2010). We aimed the above characterizes for the present study that can be used the Crs and Crp as the major makers at primary, prognostic and predicative gene expression causes in renal injury proceeding or no after Cp therapy?

In our study, the urinary and plasma creatinine, respectively, was evaluated increased in groups underwent Cp chemotherpay, showing a renal injury induced in these canines after giving remedies, nevertheless the serum creatinine increase was not significant in Cp group. Recent findings proved that creatinine did not accurately estimate the GFR attributing to the secretion and reabsorption of kidney tubule (Bellomo, Kellum, & Ronco, 2004; Premaratne et al., 2005). Compared with Crs and Crp, our data demonstrated Crs levels were more sensitive for early kidney injury detection because a more significantly dose dependent increase was detected in described groups. Therefore, we strongly recommend that renal functions should be exactly found in cases who were treated with Cp for neoplasia diseases, even without pre-clinical and post-clinical symptoms.

It is obvious from the present study that administration of a single dose of CP (7.5 mg/kg i.p.) resulted in p tubule cell necrosis, interstitial poly and mononuclear inflammations, and glomerular congestion and atrophy, to warp up, microscopic features revealed that the CP group has significant structural damage compared to the control group. According to this research, renal function biochemical parameters such as creatinine was markedly elevated in CP-treated dogs compared to control group, reflecting early damage of the filtration barrier (*i.e.,* the fenestrated capillary endothelium, GBM, and visceral epithelial podocytes). Our data are in agreement with previous reports in murine models (Abdelmegmd, Chmaisseand, & Abou Zeinab, 2010; Morigi, Imberti, & Zoja, 2004). Hemin significantly suppressed the increases in serum creatinine, which may be due to improvement in glomerular filtration damage induced by CP. Furthermore, the most researchers at different fields of Cp treatment confirmed above data such as, Shalaby, Rashed, Ismaail, Madkour, and Elwakeel (2014) obtained that a single high dose injection (5 mg/kg BW) of cisplatin in rat caused increase in sCr level showing induction of acute renal failure (Shalaby et al., 2014). Our results were in contrast with this described data, because in the present study administration of a single low dose due to a decrease in sCr biomarker expression but in parallel with it, uCr protein exhibiting an increase of this factor as a predictive indicator for proximal tubular damages such as tubule cell necrosis ,in which data is consistent with Miller, Tadagavadi, Ramesh and Reeves (2010) ,who propose that cisplatin may additionally directly induce necrosis of kidney tubular cells (Miller et al., 2010).

To improve our knowledge, there are little and /or few studies in the literature describing Crs and Crp as a biomarker of cisplatin drug induced kidney damages in dogs with solid tumors. Therefore, new randomized studies are needed to determine the true predictive and prognostic value of Crs and Crp for

dogs with solid renal cancer treated with cisplatin and the cutoff points for each Crs and Crp levels in this specific disease and each clinical outcome.

Conclusion

In summary, our data demonstrate that Crs and Crp gene expression represents a novel, sensitive, noninvasive urinary biomarker for renal solid tumors. It will be important in future translational work to examine the expression of creatinine in the urine and serum of patients with mild and early forms of acute and chronic renal injury. Furthermore, an early increase in uCr excretion may help in identifying patients at risk of cisplatin-induced renal injury who might benefit from innovative treatments to prevent cisplatin nephrotoxicity.

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