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RESEARCH ARTICLE

RETINOL BINDING PROTEIN AS BIO-MARKER FOR DIAGNOSIS OF CHRONIC KIDNEY DISEASE IN DOGS

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The present assays to diagnose kidney injurywithcreatinine/BUN measurement can only detect this long time after the onset of kidney injury. The aim of our study was to detect urinary low molecularweight (LMW) protein for early diagnosis of renal disease. The dogs were clinically screened for renal disease and separation of the urinary proteins by SDS-PAGE revealed low molecular weight proteins at Mr~36.5 KDa, 21.9 KDa, and 14.4 KDa. Western blotting revealed the presence of the retinol binding protein (RBP) among the LMW proteins.RBP appears to be a specific, non-invasive bio-marker for diagnosing dogs with renal failure.

Introduction:-

Low molecular weight proteins in urine (Simone Forterre et al., 2004) are helpful for assessment of changes in proximal tubular action before any other markers such as proteinuria or serum creatinine(Nosadini et al., 2011). Retinol-binding protein is a low-molecular-weight protein of 21 kDa that passes freely through glomerular membranes and is reabsorbed and catabolised in the proximal tubular cells (Raila*et al* 2010 andSmets*et al* 2010). Hence, retinol-binding protein (RBP) is not excreted in the urine in normal conditions (Kirsztajn, et al., 2002). Its presence in urine indicates proximal tubule injury and/or impaired proximal tubular function. However, elevated excretion of RBP can also indicate conditions like tubular damage associated with renal tubulointerstitial nephritis, tubular toxicity due to nephrotoxic drug exposure (Sun Young Kim and AreeMoon, 2012) etc. Further, Glomerulonephropathies are also associated with coexisting tubular injury, resulting in elevated RBP excretion. Changes in the excretion of retinol-binding protein (RBP) (Nabity et al., 2012 and Raila et al., 2010) appeared to be of clinical relevance in the diagnosis of canine kidney diseases.

Our hypothesis for this study is that chronic kidney disease (CKD) causes low proximal reabsorption of LMW proteins resulting in the urinary excretions of these proteins, including RBP. To test this hypothesis, we separated the urinary proteins by SDS-PAGE and detected the presence of uRBP by western blotting with anti-RBP antibodies(Maddens *et al.,* 2010, Smets*et al*., 2010 and van Hoek*et al.,* 2008). Our results indicated that the urinary excretion of LMW proteins was positively correlated with CKD and uRBP can be a biomarker for the detection of CKD in dogs.

Materials and methods:-

Clinical screening

The dogs with different agewere clinically screened for kidney disorder with routine blood-biochemical parameters and ultrasound examination of the kidneys. Healthy dogs and dogs that have elevated BUN/Creatinine levels requiring dialysis were included in the study. The urinary proteins of healthy dogs were compared with dogs with renal disease by SDS-PAGEfollowed by western blotting with RBP antibody.

Chemicals:-

Chemicals for SDS-PAGE, molecular weight markers, acrylamide N, N9-methylene bisacrylamide, ammonium persulfate, and tris (hydroxymethyl) aminoethane (Tris), enhanced Chemiluminescence (ECL) kit were from Bio-Rad, USA unless otherwise specified. Polyclonal RBP antibodyand peroxidase-conjugated secondaryantibody were obtained from Thermo Scientific, USA.

Isolation of urinary proteins:-

Urine sampleswere centrifuged within 30 minutes of collectionat 4,000 rpm at 4°C for 10 minutes to remove insoluble materials and proteins were extracted and isolated as described previously (Lau et al., 2009). The samples were then stored immediately at -20ºC prior to their separation by SDS-PAGE.

Western blotting:-

The proteins concentrations were measured by Bradford method and separated by 10 % SDS–PAGE under reducing conditions (Laemmli, 1970). For the western blot with anti-RBP antibody, the proteins were electro-transferred on to PVDF membranes. The non-specific binding sites on the membrane were blocked by incubating in TBST [20 mMTris, pH 7.4, 0.9% (w/v) NaCl, and 0.1% (v/v) Tween-20] containing 5% (w/v) dry skim milk for 1 hour at room temperature. The membranes with polyclonal anti-RBPantibody were incubated for 90 minutes at room temperature. After three 10 min washes in TBST, the membranes were incubated in the presence of corresponding HRP-conjugated secondary antibody for 1 h at room temperature and washed again. The luminescence from using Clarity western ECL blotting substrate kit (Bio-rad Laboratories, USA) was detected and images were captured digitally by using ChemiDoc MP (BioradInc, USA).

Results:-

Separation of proteins in SDS PAGE:-

The separation of urinary proteins on SDS PAGE followed by staining with coomassie brilliant blue stain showed a differential expression of proteins. Low molecular weight (LMW) proteins appeared in the urine of dogs with renal failure compared to controls. Analysis of protein bands with Image Lab software (Bio-rad Laboratories, USA) revealed protein bands at molecular weights Mr~36.5 KDa, 21.9 KDa, and 14.4 KDa (Figure 1, Lane 4) when compared to controls (Figure 1, Lanes 1-3).

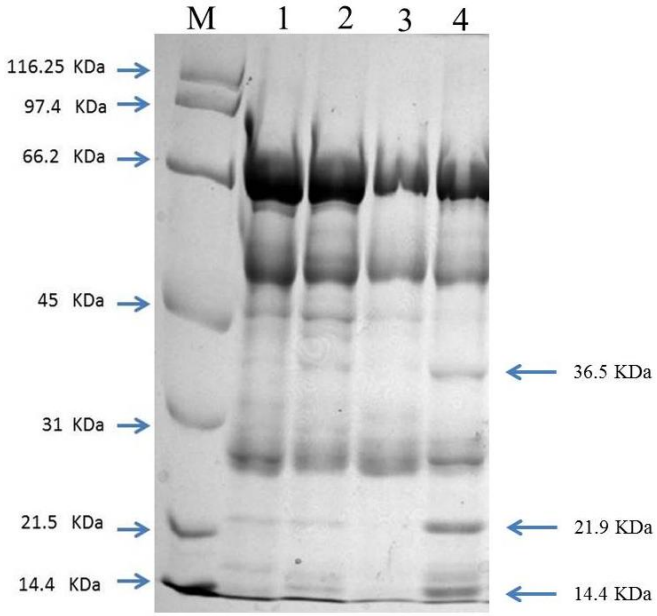

Figure 1.Separation of urinary proteins on SDS-PAGE from healthy (Lanes 1 to 3) and dogs with renal failure.Coomassie brilliant blue staining has revealed the presence of LMWurinary proteins in dogs with renal failure (Lane 4) compared to controls (lanes 1 to 3). The experiment was repeated six times and representative photo is shown.

Western blotting:-

The urinary proteins extracted were electro-transferred onto PVDF membrane for western blot. Probing the membranes with anti-RBP antibody revealed that the retinol binding protein appeared only in urinary proteins from dogs with renal failure (Figure 2, Lane 3) compared to controls (Figure 2, Lanes 1 and 2)

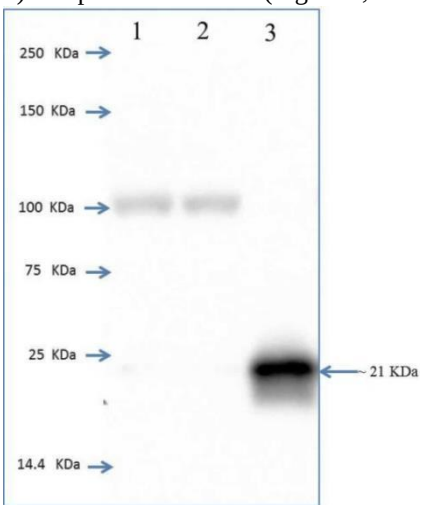

Figure 2.Western blots of urinary protein (15µg/lane) probed with anti-RBP antibody. Note that there is a marked presence of RBP at Mr~ 21 KDa in dogs with renal failure (Lane 3) compared to controls (Lane 1 and 2). The experiment was repeated six times and representative photo is shown.

Discussion:-

The present tests for diagnosis of chronic renal disease with elevated creatinine and blood urea nitrogen (BUN). However, approximately 65-75% of kidney function must be lost to see an increase in serum creatinine or BUN (Lefebvre, 2011). Measuring the changes in proximal tubular action can detect renal disease before any other markers such as proteinuria or serum creatinine (Nosadini et al., 2011).Our experiments show that there differentially expressed proteins are present in the dogs with renal failure at the molecular weights of Mr~36.5 KDa, 21.9 KDa, and 14.4 KDa (Figure 1, Lane 4), confirming the earlier studies that low molecular weight proteins in urine (Simone Forterre et al., 2004) can be useful biomarkers for diagnosis of renal disease. Further, our western blotting study with RBP has shown that it can serve as useful biomarker to detect renal failure. This RBP is not present in the urine of healthy dogs (Figure 2, Lanes 1 & 2) whereas the same is present in large quantities in the urine of the dogs with renal failure (Figure2, Lane 3). Probably, this RBPfiltered through glomerular membranesare not reabsorbed by renal proximal tubules cells. Our studies are similar to report that RBP is not excreted in the urine normal conditions (Kirsztajn, et al., 2002) and age did not appear to affect uRBP/c (Smets*et al.,* 2010). Its presence in urine indicates proximal tubule injury and/or impaired proximal tubular function (D'amico and Bazzi 2003) or extent of interstitial fibrosis in the kidney (Nicolas et al, 2014).In dogs with X-linked hereditary nephropathy, a model of progressive proteinuric nephropathy, uRBP/c correlated most strongly with serum creatinine, GFR, histologic lesions, and demonstrated progressive increase in values compared to other markers of renal function, even in late stage kidney disease (Nabity*et a*l 2012 and Vinge*et al* 2010. Our studies conclusively prove RBP as biomarker in the diagnosis of chronic kidneydisease. Further studies are being done to identify the rest of urinary proteins in dogs with renal failure and validate them for their diagnostic importance.

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