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Nephrology Section

Peptiduria in patients with acute renal failure

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ABSTRACT

Introduction: Proteinuria is a common finding in acute renal failure (ARF). Recently, there is an increasing interest in knowing the significance of peptiduria in renal failure patients. The current study has been undertaken to know the levels of peptiduria in ARF patients.

Method: 58 ARF patients and 55 healthy controls were selected, based on inclusion and exclusion criteria. Urinary proteins and peptide levels were determined by the spectrophotometer based Lowry and Bradford methods.

Results: The urinary %s of peptides in urine were significantly decreased in ARF patients; there was a wide variation in the levels of peptides in grams per liter of urine and in the levels of peptides in grams per gram of creatinine. Urine creatinine levels correlated positively with the urinary peptide levels.

Conclusion: ARF patients showed a significant decrease in the %s of urinary peptides and the %s of the urinary peptides seemed to be a more appropriate measure in determining the levels of urinary peptides. To study the significance of urinary peptides in the diagnosis and management of ARF needs future research in this field.

Key Words : Peptiduria, Proteinuria, Acute renal failure, % Urinary peptides

INTRODUCTION

Acute renal failure (ARF) is characterized by a sudden or gradual decline in the glomerular filtration rate (GFR), a slow and steady accumulation of nitrogenous waste products, and an inability of the kidney to regulate the balance of sodium, electrolytes, acid, and water [1]. The ischaemic damage in ARF is generally most severe in the early proximal tubule (S3 segment) and the thick ascending limb of the loop of Henle [2]. Poor oxygenation leads to a variety of secondary factors that promote the development of tubular injury, including the intracellular accumulation of calcium, the generation of reactive oxygen species, the depletion of adenosine triphosphate, and apoptosis [2-4]. Many tubular enzymes have been studied as the markers of the necrotic/apoptotic damage or the dysfunction of (proximal) the tubular cells. Three major origins have been identified: the lysosomes, the brush-border membranes, and the cytoplasm of the cells [5, 6].

Several studies have demonstrated that increased urinary amounts of enzymes are useful to detect acute tubular damage at a very early stage, but increased enzymuria may also be induced by a reversible mild dysfunction of the cells, which is not necessarily associated with irreversible damage. The usefulness of enzymuria may be obscured by the low threshold for the release of tubular enzymes, even in response to injury that may not proceed to ARF [7]. However, enzymes are also released during chronic glomerular diseases, which might limit their use as a marker of tubular injury only [8-11]. Some of the best-characterized tubular enzymes which can be used to detect tubular injury are glutathione-S-transferases (GSTs), γ-glutamyl transferase (γ-GT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG), fructose-1,6-biphosphatase, and Ala-(Leu-Gly)-aminopeptidase [8, 9]. The increased urinary excretion of these proteins implies tubular injury.

The low-molecular weight proteins that escape complete reabsorption when the proximal tubular cells are overloaded or damaged have been used as markers of the damage or dysfunction of these cells [5]. Some of the best-characterized tubular proteins which can be used to detect proximal tubular injury are α1- and β2-microglobulin, retinol-binding protein, and cystatin C [10][11] [9]. The principle barrier to the passage of blood proteins has been thought to reside in the glomerular capillary wall. The restrictive permeability of the glomerular filter to macromolecules has been attributed to exclusion, based on their sizes, configurations, and electrical charges [12]. Previously, it was believed that most of the filtered protein that reaches the renal tubule is degraded and entirely reabsorbed into the blood stream [13].

However, recent studies suggest that in humans, 95% of the albumin is reabsorbed from the proximal tubules and are, degraded to produce small peptides (<10 kD) that are excreted in urine [14]. Russo et al suggested that the albumin degradation products are excreted as peptides in urine and that the quantities of these peptides we are in great excess of intact albumin in normal individuals [15]. The exact anatomical location of the degradation pathway has not been determined;, it is likely to take place in cells which are distal to the glomerular basement membrane, most probably in the tubular epithelial cells, where albumin is subjected to endocytosis and is trafficked to the lysosomes. Once degraded, the small peptides resulting from albumin degradation small peptides are subjected to exocytosis into the tubular lumen and are excreted in the urine [16-19].

We have selected some ARF patients to determine the levels of the levels of urinary peptides and to compare d them with that of healthy individuals to see the difference in the excretion of these peptides.

MATERIALS AND METHODS

Fifty eight subjects with ARF were selected as the cases. Fifty five healthy controls were participated included in this study. The inclusion criteria for the ARV cases were: age > 18 years, ARF of any aetiology, defined by a more than 30% rise in serum creatinine from the baseline, patients with renal failure presenting to the hospital for the first time with a short history (< 3 months duration), and ultrasound showing normal sized kidneys (> 8.5 cm). The exclusion criteria were: age <18 years, obstructive acute renal failure, patients with a preexisting history of renal failure (acute on chronic renal failure), patients with a history of diabetes mellitus or hypertension, kidney size < 8.5 cm on ultrasound or the evidence of hydronephrosis and, patients presenting as sepsis with acute renal failure. Healthy controls aged more than 18 years, with no past or present history of any medical illness, those who were not on any kind of medication and, those who were non-smokers and; non-alcoholics were included in the study.

Twenty four hour urine samples from the 58 ARF cases and the 55 healthy controls were as collected in a brown bottles containing toluene as the urine preservative; the urine sample bottles with the urine were as stored at 4°C during the period of collection. The samples were centrifuged at 3000 rpm for 10 minutes and were analyzed immediately after the collection period. Informed consent from the subjects who were involved in the study and ethical clearance from the institutional review board were as taken.

REAGENTS

Special chemicals like the Biorad reagent and, bovine serum albumin (BSA) were obtained from Sigma Chemicals, St Louis, MO, USA. All other reagents were of analytical grade.

Protein stock: BSA was dissolved in phosphate buffered saline. For the preparation of the standard graph, BSA was used in different concentrations; for the Bradford assay: it was used in concentrations of 2, 4, 6, 8, and 10 µg/ml; for the Lowry assay: it was used in concentrations of 50, 100, 150, 200, and 250 µg/ml.

For The Lowry assay: we have slightly modified the Lowry's assay for determining total urinary proteins. The set of Lowry's reagents were prepared as; reagent A: 2% sodium carbonate, reagent B1: 1% sodium potassium tartarate, reagent B2: 0.5% $CuSO_4$ in reagent B1, reagent C: 50 ml reagent A + 1 ml reagent B2, and reagent D: 1 N Folin Ciocalteau reagent.

METHODS

The proteins and peptides in urine were measured by using a Genesys 10UV spectrophotomter. The urine creatinine levels were determined by using a clinical chemistry automated analyzer (Hitachi 912).

Both the Lowry and the Bradford assays were done after suitably diluting the urine samples. Urinary proteins, together with the peptides, were measured by using Lowry's assay [20]. The urinary proteins were determined by using the Bradford assay [21]. The urinary peptide levels were determined by subtracting the Bradford's value from the Lowry's value in the same urine sample (Lowry value – Bradford value). All calculations were done by using separate calibration curves which were prepared for each method.

For the Lowry estimation, 0.2 ml of diluted urine sample was taken in two sets of eppendorf tubes (1 and 2). 0.2 ml of 145 mM NaCl was taken in another tube and was labeled as the reagent blank (RB). To RB and set 1, 1 ml of reagent C was added to the RB and set 1. To set 2, 1 ml of reagent A was added to set 2. The tubes were shaken vigorously and were incubated for 10 minutes at room temperature. Reagent D was added at the end of 10 minutes and the tubes were vortexed. The vigorous shaking is crucial for colour development. The tubes were incubated at room temperature for 30 minutes and the absorbance was read at 600nm.

After correcting for the respective blanks, the absorbance values of set 2 were subtracted from its their counterparts in set 1. The difference in the readings is was because of the copper pre-treatment of set 1. The total protein content was calculated from the calibration curve. After multiplying with the dilution factor, the total protein content, including the peptides, was expressed in grams per milliliter (gm/ml) and in grams/gram of urine creatinine.

For the Bradford assay, 1 ml of diluted urine sample was added to 1 ml of Biorad reagent. 1 ml of PBS was added to the Biorad reagent, which served as the reagent blank. The contents were mixed and incubated at room temperature for 30 minutes and the absorbance was read at 595 nm. The protein content in the sample was calculated by using the calibration curve, and after multiplying with the dilution factor, the values were expressed in grams/ml and grams/gram of urine creatinine.

The total urinary peptide content in the sample was calculated by subtracting the Bradford's value (protein content) from the Lowry's value (total protein including peptides), and the peptide content in urine was expressed as grams/L and grams/gram of urine creatinine. Because peptide excretion may depend on the filtered load of the urinary proteins (which cannot be determined directly), hence we have calculated the peptide values as the percentage of total protein material in urine (an indirect measure of the filtered load: [Lowry – Bradford]/Lowry x 100) against proteinuria, and expressed it as % urinary peptides.

STATISTICAL ANALYSIS

All statistical analyseis was were done by using the statistical package for social sciences (SPSS), version 16. The independent sample t test and the Mann Whitney U test was done were used to compare the mean values. A Pearson's correlation was used to correlate between the parameters. P values <0.05 were as considered to be significant. Microsoft Office Excel was used to prepare the correlation figures.

RESULTS

As depicted in t [Table/Fig-1], we have found significant change in the urinary peptides in the ARF patients as compared to the healthy controls. The total protein content (protein by the Lowry's method) in the ARF patients' urine was found to be higher than that

	Healthy Controls (n = 58)	Acute Renal Failure Cases (n = 55)
Lowry's method (gm Proteins/L)	2.95±0.22 Min: 0.55, Max: 6.40	15.02±1.99* Min: 0.78, Max: 57.38
Bradford's method (gmProteins/L)	0.06± .005 Min:0.00, Max: 0.14	1.54±0.32* Min:0.08, Max: 13.50
Gm Urinary Peptides/L (Lowry – Bradford)	2.88±.22 Min:0.47, Max: 6.32	13.48±1.89* Min:0.36, Max: 55.10
Gm Urinary Peptides/gm Cr	4.13±.25 Min: 1.74, Max: 10.26	36.52±15.58 Min: 0. 82, Max: 918.25
(Lowry – Bradford) x 100 Lowry (% urinary peptides)	97.12±0.45 Min: 84.96, Max: 99.74	85.27±2.45* Min: 16.00, Max: 98.17

[Table/Fig-1]: Independent sample t test for all the determined biochemical parameters in both healthy controls and acute renal failure cases (values expressed as mean ± standard error of mean, both minimum and maximum value observed also mention).
*P <0.0001 compared to healthy controls.

of found in the controls (p<0.001) and also, there was a significant increase in the protein content (protein by the Bradford's method) (p<0.0001). The peptide content in the urine was calculated by taking the difference in the protein content between the Lowry's method and the Bradford's method (protein content by the Lowry's method – protein content by the Bradford's method). It appeared that there was a significant increase in the urinary peptide content of the ARF patients as compared to that of the healthy controls (p<0.0001). However, on taking the filtration load into consideration, where the % peptides a were calculated, we have seen found a significant decrease in the urinary peptide content in the ARF patients as compared to that of the healthy controls (p<0.0001).

We have observed significant skewed values in all the parameters that we had ve determined (mentioned in Table/Fig-1 as minimum and maximum values). Because of a wide variation in the observed parameters, we have also analyzed the above parameters by the Manny Whiney rank sum test. As mentioned in [Table/Fig-2], there was a significant difference in the observed parameters in the ARF patients as compared to the healthy controls (p<0.0001). We have observed that the urinary peptides/gm of creatinine, which showed no significant change between the two groups by the independent sample t test, has showed a significant difference (p<0.0001) by the Mann Whitney rank sum test. On applying Pearson's correlation, we have seen observed that urine creatinine levels correlated positively with the levels of urinary peptides (r = 386, p<0.003) [Table/Fig-3].

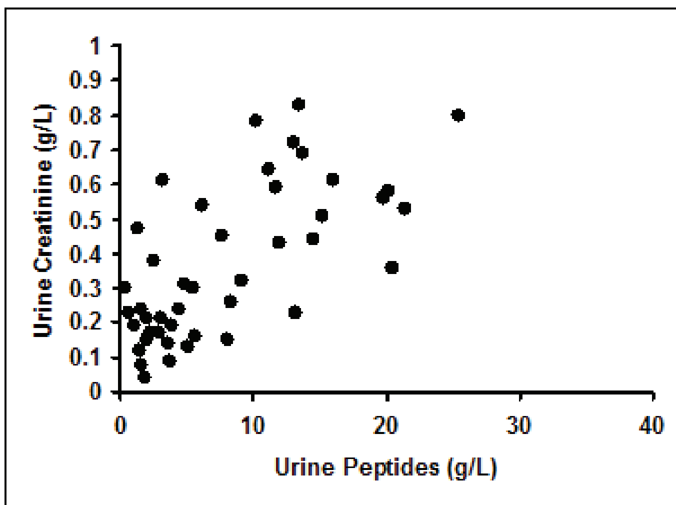
DISCUSSION

There is a current considerable current interest in the biochemical markers for the early detection of ARF, so that appropriate measures can be taken at the earliest to avoid its complications which leading to increased mortality. In recent years, intense research in this field has given suggested many biomolecules as the potential early markers of ARF. To mention, a few among them are, glutathione-S-transferase isomers (GST-α, GST-ϖ), γ-glutamyl transferase (γ-GT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG), fructose-1,6-biphosphatase, Ala-(Leu-Gly)-aminopeptidase, α1- and β2-microglobulin, retinol-binding protein, cystatin C, Na+/H+ exchanger isoform 3 (NHE-3), neutrophil gelatinase-associated lipocalin (NGAL), Cysteine-rich protein 61 (Cyr61), kidney injury molecule 1 (KIM-1), urinary interleukins and adhesion molecules (IL-18, cytoskeletal disruption-derived actin and IL-6, IL-8, IL-1, transforming growth factor β1), perforin, granzyme B, and CXCR3-binding chemokines, endothelin, proatrial natriuretic peptide and, tryptophan glycoconjugate. All these biomarkers have been proposed as early markers in the detection and management of RAF ARF, but each of them have their advantages and disadvantages in their utility as the markers of ARF, the details of which can be found in a review article by Trof RJ et al [7].

We have found a significant decrease in the levels of the urinary peptides in ARF, the urinary peptides measured as % urinary peptides, thus indicating the filtered load of protein shown significant decrease in ARF. As depicted in [Table/Fig-1], the urinary peptides which were calculated as the difference between the values from the Lowry's and the Bradford's method (Lowry protein; Bradford protein), which were expressed per liter of urine, apparently showed increased levels of peptides in ARF (mean value 13.48) as compared to that in the healthy controls (mean value 2.88). This is because of a significant variation in the observed peptide levels in the ARF patients (skewed observation, Min:0.36, Max: 55.10). A similar observation was noted when urinary peptides were expressed as grams of urinary peptides per

		Lowry's method (gm Proteins/L)	Bradford's method (gm Proteins/L)	Gm Urinary Peptides/L (Lowry – Bradford)	Gm Urinary Peptides/ gm Cr	% Urinary peptides
Mean Rank		Control: 27.40	Control: 18.60	Control:31.00	Control: 24.83	Control: 69.11
		Case: 58.834	Case: 64.14	Case: 56.66	Case: 60.38	Case: 33.66
Sum of Ranks		Control:959.00	Control: 651.00	Control: 1085.00	Control: 869.00	Control: 2419.00
		Case: 3412.00	Case: 3720.00	Case: 3286.00	Case: 3502.00	Case: 1952.00
Mann-Whitney U		329.000	21.000	455.000	239.000	241.000
Wilcoxon W		959.000	651.000	1.085	869.000	1952.000
Z		-5.440	-7.883	-4.441	-6.154	-6.138
Asymp. Sig. (2-tailed)		.000	.000	.000	.000	.000

[Table/Fig-2]: Mann Whitney Rank Sum test for the all the determined biochemical parameters in both healthy controls and acute renal failure cases.



[Table/Fig-3] : Correlation between urinary peptides and urine creatinine in acute renal failure cases

grams of creatinine[21]. This is possibly because of a significant variation in the urine creatinine levels which were used to express the urinary peptides (mean 36.52, Min: 0.82, Max: 918.25). Hence, have we used the Mann Whitney rank sum test due to a skewed observation in the measured parameters, and it has shown a significant difference in the peptide levels between the ARF cases and the healthy controls.

Of all Regarding the different forms of expressing the measured urinary peptides, in our current study, apart from our previous study in on general proteinuria cases [22], we have seen that the expression of urinary peptides as % peptides is more appropriate, and which takes into account the filtered load of protein in measuring the peptide levels. We have found a significant decrease in the % urinary peptides in the ARF cases and the determination and the expression of peptides as % urinary peptides have shown a significant difference between the ARF cases and the healthy controls when compared to the determinination g and expression of ng the peptides as gm peptides per gm of urine creatinine. In our study group, there was no difference in the gms of urinary peptides per gm of urine creatinine between the ARF cases and the healthy controls, but when the filtered load of urinary proteins was taken into consideration by using the above formula, we have found a significant difference in the % peptides that were excreted in the ARF cases as compared to the controls [Table/Fig-1].

Previous authors have reported a similar decrease in the % urinary peptides in renal disease patients [23] and in proteinuria cases [22]. We have also found that the urine creatinine levels correlated positively with the urinary peptide levels [Table/Fig-3], thus indicating that as the urine creatinine levels decreased s due to renal injury, the urinary peptides will also be decreased d due to renal paranchymal damage and the loss of the protein degradative capacity of the renal tubular cells. Previous authors have also found a significant decrease in the lysosomal enzymes in the urine of micro and macroproteinuric patients as compared to the healthy controls, thus indicating a decreased activity of the tubular lysosomal enzymes in degrading proteins [23]. In our previous study, we have found a significant positive correlation between urine creatinine and urinary peptide levels in proteinuria cases [22, 24]. This consistent finding shows some relationship between the impaired renal function and the levels of peptides that are were found in the urine of the ARF patients.

One needs to establish the specific urinary peptides or the specific cut off limits to know the initiation of renal failure. Further studies in on this aspect may throw ugh some light on the utility of the urinary peptides in the diagnosis and management of ARF.

In conclusion, there is was a significant decrease in the urinary peptide levels in patients with ARF., The measurement of the urinary peptide levels as % urinary peptides which indirectly indicate the filtered load of protein seems to be more appropriate way of expressing the urinary peptides. The significance of the urinary peptides in renal diseases needs to be determined by further research.

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