Standardization of renal function evaluation in Wistar rats (*Rattus norvegicus*) from the Federal University of Juiz de Fora's colony

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ABSTRACT

Introduction: There is great interest in the use of animal models in the study of renal pathophysiology requires standardization of parameters. **Objective:** Standardize assessment of renal function in rats from in the Center for Reproductive Biology of Federal University of Juiz de Fora's colony. **Methods:** Thirty Wistar rats were used and performed measurements of creatinine (serum and urine), serum urea and proteinuria. Were evaluated: the urine collection interval in metabolic cages (24 hours or 12 hours), the need for 12-hour fast, the need of urine and serum deproteinization for creatinine measurement, need of serum deproteinization in animals with acute kidney injury to a spectrophotometer and ELISA, and the comparison of 24-hour proteinuria (PT 24 hours) with the protein/creatinine ratio (rP/C). Means were compared by the *Student's t* test, Pearson correlation, Bland-Altman plot for agreement and linear regression model to estimate PT 24 hours from rP/C. **Results:** The 24 hours urine output was greater than 12 hours, interfering with the creatinine clearance calculation. In the fasting group showed less water intake and lower urinary creatinine. There was great variability for the deproteinized whey and readings performed in the two devices were similar. There was a strong correlation between PT 24 hours and rP/C and the equation was generated: PT 24 hours = (8.6113 x rP/C) + 1.0869. **Conclusion:** Was standardized: 24-hour urine collection without fasting. The deproteinization showed no benefit. The measurements were performed with spectrophotometer reliability. It generated a practical formula for estimating PT 24 hours through rP/C.

Keywords: creatinine; kidney function tests; proteinuria; rodentia; specimen handling.

INTRODUCTION

Animal experimentation is an important tool for understanding the pathophysiology of several diseases; considering it leads to the discovery and development of breakthrough treatments, methods of prevention and diagnosis - relevant for human health.[1] The use of animals, while respecting ethical precepts, overcomes existing limitations on human experimentation and provides for rapid investigation of diseases, in an efficient and low cost manner.[2]

Chronic kidney disease (CKD) carries high morbidity and mortality and it has had a progressive increase in incidence and prevalence in recent years. Between 2000 and 2010, there was an increase of 115% in the number of dialysis patients in the country, totaling 91,314 patients in 2011.[3] CKD is characterized by the presence of kidney injury, which can be evidenced by a decrease in kidney function assessed by glomerular filtration rate (GFR), or proteinuria, both persisting for three months.[4] In Brazil, according to the Census of the Brazilian Society of Nephrology in 2009, more than 77,000 patients started hemodialysis as replacement therapy for kidney function,[3] with Health System expenses accounting for more than 1.5 billion Reals in the same year.[5,6]

The concern with the increasing prevalence and severity of CKD

encourages research involving animal models, with the aim of finding alternatives for treating the disease, improving patients' quality of life and reducing their mortality. The experimental protocols for using these models should be well defined and made from prior knowledge of the procedures and parameters to be investigated.[2] Such practices are critical to the reliability of the experimental results, so that the analysis and interpretation of the data enable its use for the benefit of human beings.[7]

The laboratory test most commonly used to measure kidney function is taken from the GFR, which can be accomplished by methods considered the gold standard, but involving injection of drugs and their excretion.[8] In clinical practice and animal experiments, the GFR is estimated by methods which rely on measurements of urinary creatinine, serum creatinine and 24h urine flow.[9] Another measure widely used in the evaluation of renal function is proteinuria quantification - urinary excretion of protein - which can be evaluated by the 24h microalbuminuria technique, 24-hour proteinuria (24h PT) or by the protein/creatinine ratio (rP/C) or albumin/creatinine from urine-isolated samples.[10] To better meet the study's objectives it is necessary to adapt the procedures and techniques used to measure these parameters and set benchmarks for normal animals.[11] These values contribute to the assessment of pathological changes and help evaluate the results obtained from experimental procedures.[12]

Considering the interest in experimental models of kidney disease and the need to expand biological standardization studies involving animal models; this study was carried out in order to standardize the assessment of kidney function in rats (*Rattus norvegicus*) from the animal house colony of the Center or Reproduction Biology (CBR) of the Federal University of Juiz de Fora (UFJF).

METHODS

ANIMALS

We used 30 male rats (*Rattus norvegicus*) of the Wistar lineage with 8 to 12 weeks of age. The animals were obtained from the animal house of the UFJF CBR, where the experiments were conducted. We kept the animals in polypropylene cages, provided with wood shavings bedding and housed in air-conditioned shelves (Alesco Indústria e Comércio Ltda., Monte Mor, Brazil). Water and pelleted food (Nuvilab-CR1, NUVITAL Nutrients Veterinary Products Ltd., Curitiba, Brazil) were offered ad libitum and room temperature was maintained at around 22 °C, with automatically controlled lighting to light up at 6 am and off at 6 pm. In the metabolic cages, the ration was offered in powder, according to the study design.

The procedures comply with federal law 11.794 of October 8, 2008, with the regulations from the National Board of Animal Control and Experimentation and were approved by the Ethics Committee on Animal Experimentation of the Federal University of Juiz de Fora, as Protocol # 008/2011.

EXPERIMENTAL DESIGN

The animals were randomly divided into five groups, 24-hour urine collection Group (G24, N = 6); 12-hour urine collection group (G12, N = 6); 12-hour fasting Group (JE12, N = 6); Deproteinization Group (D24, N = 6) and Kidney Injury Group (IR24, N = 6). The study was divided into four experiments: Urine collection; Fasting, Deproteinization and Analysis Methods:

URINE COLLECTION

This experiment was used to determine the range of urine collection and assess the need to keep animals for 24 hours in metabolic cages. We collected the urine from the animals in the G24 group for 24 hours, starting at 08 pm and ending at 08 am the next day, the animals received standard diet at libitum. Considering the nocturnal habits of the *Rattus norvegicus* species, the G12 group of animals was kept in metabolic cages for 12 hours during nighttime, from 8 pm to 08 am of the next day, also receiving the standard diet ad libitum. In order to prove the predominance of animal activity during the night and that the characteristics of

the 24-hour urine are the same as the 12-hour urine, we tested urine creatinine and proteinuria in groups G24 and G12, and compared them. We calculated 24h and 12h creatinine clearances, when necessary.

Fasting

To establish the need for fasting blood collection, the animals in the JE12 group were fasted for 12 hours during their stay in the metabolic cages, starting at 8 pm and ending at 08 am the next day. We tested serum creatinine and urea from groups G12 (non-fasting) and JE12. To evaluate the interference of leftover food in urine collectors, we determined the concentrations of urinary creatinine and proteinuria in the JE12 group and compared with the G12 group. We also calculated the 12h creatinine clearance.

Deproteinization

This experiment evaluated whether there was a need to deproteinize the urine samples collected in the aforementioned experiments (G24, G12 and JE12), and also of the serum samples collected in the experiments above, added to the serum samples from the D24 group, a total of 24 animals. The mean plasma levels of creatinine were compared in samples with and without deproteinization,[13] along with the calculations of creatinine clearance using the two samples.

Analysis methods

Standardizing the creatinine dosing method

In order to evaluate the need to use a more accurate test reading method (ELISA reader - Enzyme-Linked Immunosorbent Assay) in the sera of animals with kidney dysfunction, we measured serum creatinine with and without deproteinization of the IR24 group with two devices: automatic analyzer by the spectrophotometric method (LabMax Progress, Labtest Diagnostica SA, Lagoa Santa, Brazil) and the ELISA reader (R&D Systems, Minneapolis, USA). In the IR24 group we induced acute kidney injury (AKI) by the cisplatin experimental model.[14] After 3 days using cisplatin, we collected blood to measure creatinine levels.

Urine collection, biological parameters and intake

We measured total diuresis volume from the urine collection after the animals stayed in the metabolic cages. All urine samples were centrifuged at 3,000 rpm for 10 minutes in an automatic refrigerated centrifuge (Sorvall, Suwanee, USA). The samples were placed in cryogenic tubes (Eurotips Scientific, São Paulo, Brazil) and kept at -8 °C until the analysis performed on the same day of collection. Prior to blood collection we evaluated: the animal weight on an analytical scale, the amount of feed consumed and the volume of water intake through the leftovers in the metabolic cage containers for feed and water.

Blood collection for biochemical analysis

For blood collection, the animals were anesthetized with ketamine (König SA, Avellaneda, Argentina) at a dose of 90 mg/kg and xylazine (König SA, Avellaneda, Argentina) at a dose of 10 mg/kg, intraperitoneally injected. Blood was collected from a cardiac puncture[15] and centrifuged at 3,000 rpm for 10 minutes in an automatic refrigerated centrifuge (Sorvall, Suwanee, USA) to separate the serum and package it in cryogenic tubes. The samples were kept under refrigeration (2 °C to 8 °C) until the time of analysis, performed on the same day of collection. After heart puncture, the animals underwent diaphragm rupture as a supplementary euthanasia procedure.

Deproteinization procedure

For deproteinization, 100 µL of the sample were diluted with 200 µL of distilled water. To the solution we added 100 µL of sodium tungstate (Vetec, Duque de Caxias, Brazil) and 10% m/v 100 µL of sulfuric acid (Vetec, Duque de Caxias, Brazil) at 1.84% v/v. After homogenization in a Vortex-type shaker (Cole Parmer, Chicago, USA), the solution was centrifuged for 10 minutes at 9,000 rpm in a microcentrifuge (Fanem, São Paulo, Brazil) and then the supernatant was removed and used for analysis.[16,17]

AKI induction

AKI was induced by intraperitoneal administration of cisplatin 10 mg/kg (Faulding, Rio de Janeiro,

Brazil).[14] Kidney function was assessed by measuring serum creatinine, three days after the drug administration.

BIOCHEMICAL ANALYSIS

The equipment used for all biochemical analyzes was the LabMax Progress® (Labtest Diagnostica SA, Lagoa Santa, Brazil). Other evaluations of serum samples with and without deproteinization of the IR24 group was performed in an ELISA reader (R & D Systems, Minneapolis, USA) with a of 520 nm wavelength filter.

ESTABLISHING THE CREATININE CONCENTRATION

The creatinine concentration, both in serum and in urine (1:25 dilution) were measured using the Creatinine K® commercial kits (Labtest Diagnostica SA, Lagoa Santa, Brazil), which uses a two-point optimized kinetic procedure based on the modified-Jaffe reaction.[18] For dosing purposes, 50 µL of the serum sample or the diluted urine was added to 50 µL of alkaline picrate, mixed and aspirated into the automatic analyzer bucket set to zero at 510 nm, and then we measured the absorbance at 30 and 90 seconds. The results were expressed in mg/dL; and for urine they were corrected by the dilution factor.

DETERMINING SERUM UREA CONCENTRATION

For determining serum urea, we used the Liquiform® Urea UV test (Labtest Diagnostica SA, Lagoa Santa, Brazil) which uses an enzymatic system by UV photometry and two-point kinetics. Urea is hydrolyzed by urease, producing ammonia and carbon dioxide. The ammonia reaction reduces absorbance, which is proportional to the urea concentration in the sample. 10 µL of serum were aspirated into the photometer reservoir previously adjusted to 340 nm, and then we measured the absorbance at 30 and 90 seconds. The results were calculated from the absorbance difference between the two time periods and expressed in mg/dL.

DETERMINING PROTEINURIA

To determine the proteinuria, we used the Sensiprot® test (Labtest Diagnostica S.A., Lagoa Santa, Brazil), through the end point reaction, in which 50 µL of the diluted urine sample (1:5) is mixed with 100 µL of color reagent containing pyrogallol. The pyrogallol red reacts with the sodium molybdate, forming a complex which when combined with the protein an acid medium develops a blue colored chromophore. Proteinuria quantitation was made using a spectrophotometric method based on the 600 nm absorbance relationship between the sample and the standard solution (50 mg protein/dL). Proteinuria concentration results were obtained in mg/dL and corrected by the dilution factor. 24-hour PT concentration was expressed in milligrams (mg), calculated from the product of its concentration and the total volume of 24 hours in dL. The 24-hour PT value for the groups that remained for 12 hours in a metabolic cage was calculated using the value of diuresis multiplied by two.

Apart from the proteinuria value measured, 24-hour PT was estimated from the ratio of protein concentration and the creatinine concentration, measured in the same urine sample from each animal.[19] We then correlated the proteinuria measurements obtained for each animal.

STATISTICAL ANALYSIS

The data was expressed as mean and standard deviation. The Skewness and Kurtosis tests were used to assess variable distribution normality. The mean values were compared by the *Student's t*-test. The degree of association between variables (conventional and deproteinized samples) was determined by the Pearson's correlation coefficient, and the degree of agreement was assessed by the Bland-Altman test. The linear regression model was used to calculate the correction factor based on the average ratio between the variables: rP/C and 24-hour PT, the degree of association between the two was determined by the Pearson's correlation coefficient and the degree of agreement was assessed by the Bland-Altman test. All statistical calculations were performed using the SPSS 13.1® (SPSS Inc. Chicago, IL, USA) software and statistical significance was established at $p < 0.05$.

Results

In the urine collection experiment, urine volume and the mean creatinine clearance of the G12 group were lower when compared to the G24 group. All other parameters did not differ between the two groups (Table 1).

The fasting experiment compared the results from the G12 group and those from the JE12 group; the later remained fasting for 12 hours for urine collection in metabolic cages. Regarding consumption, there was a decrease in water intake among the fasting animals. The mean urine volume was higher for these animals (Table 2).

The mean plasma levels of serum creatinine and urea from the G12 and the JE12 were not statistically different. However, the creatinine values in the urine of animals from the G12 group were higher, while the values of creatinine clearance were lower compared to the values presented by the animals in the JE12 group. This is due to the interference of serum creatinine, which was lower in the JE12 group, even without statistical difference. The difference between 24-hour PT values was significant; however, there was no rP/C difference in both groups (Table 2).

In the deproteinization experiment we used 18 urine samples collected from animals in groups G24, G12 and JE12. For serum evaluations we used 24 samples from groups G24, G12, D24 and JE12. All samples were analyzed without the deproteinization procedure (conventional sample) and with the deproteinization process (deproteinized samples). There was no statistical difference between the value of urine creatinine and creatinine clearance using samples with and without deproteinization (Table 3). There was a strong correlation among urine creatinine values (r = 0.957; $p < 0.001$) (Figure 1A). Using the Bland-Altman graph we also did not find evidence of bias when comparing the conventional urine creatinine and creatinine in deproteinized urine, since the individual data was randomly distributed above and below the mean central curve (Figure 1B).

The mean serum creatinine values were not different between the conventional samples and the deproteinized ones, which showed great variability (Table 3). Creatinine values were in agreement with each other (r = 0.501, $p = 0.013$) (Figure 1C); however, according to the Bland-Altman analysis, there was evidence of bias in the comparison between conventional serum creatinine and creatinine in the deproteinized serum (Figure 1D).

Due to the discrepancy in serum creatinine concentrations in deproteinized samples, new measurements were performed in a group with AKI, which would knowingly present elevated serum creatinine. This experiment was called Analyses Methods, in which two analyzes were performed: one in an automatic analyzer by the spectrophotometric method and the other in an ELISA reader. We lost two animals from the IR24 group. There was no statistical difference between the conventional samples and the deproteinized ones in any of the equipment utilized (Table 4). There was also no correlation: Samples of conventional and deproteinized sera analyzed by spectrophotometry (r = 0.713, $p = 0.287$), and

TABLE 1	PARAMETERS ASSESSED IN GROUPS G24 AND G12, IN THE URINE COLLECTION EXPERIMENT (COMPARING THE 24-HOUR AND THE 12-HOUR COLLECTION TIMES)		
	G24 Group 24-hour urine N = 6	G12 Group 12-hour urine N = 6	p
Weight (g)	235.2 ± 28.8	237.0 ± 24.4	0.91
Feed intake (g)	19.7 ± 3.3	16.9 ± 2.6	0.14
Water intake (ml)	23.5 ± 7.3	19.5 ± 10.2	0.45
Urine volume (ml)	10.9 ± 2.2	4.7 ± 1.4	< 0.001
Urine creatinine (mg/dL)	88.5 ± 11.1	97.4 ± 19.6	0.36
Serum creatinine (mg/dL)	0.50 ± 0.07	0.62 ± 0.02	0.009
Creatinine clearance (ml/min)	1.32 ± 0.2	0.98 ± 0.2	0.02
24-hour proteinuria (mg/dL)	8.8 ± 5.3	10.4 ± 4.6	0.59
Protein/creatinine ratio	0.92 ± 0.6	1.16 ± 0.4	0.43

TABLE 2	PARAMETERS ASSESSED IN GROUPS G12 AND JE12, IN THE FASTING EXPERIMENT, WHICH COMPARES THE NEED FOR A 12-HOUR FASTING		
	G12 No fasting N = 6	JE12 12-hour fasting N = 6	*p*
Weight (g)	237.0 ± 24.4	245.9 ± 17.5	0.48
Water intake (ml)	19.5 ± 10.2	5.8 ± 2.6	0.01
Urine volume (ml)	4.7 ± 1.4	7.9 ± 2.6	0.02
Urine creatinine (mg/dL)	97.38 ± 19.61	67.28 ± 23.79	0.04
Serum creatinine (mg/dL)	0.62 ± 0.02	0.53 ± 0.09	0.07
Creatinine clearance (ml/min)	0.98 ± 0.17	1.29 ± 0.22	0.02
24-hour creatinine (mg/dL)	10.4 ± 4.6	15.2 ± 2.0	0.05
Protein/creatinine ratio	1.2 ± 0.4	1.6 ± 0.1	0.07
Urea (mg/dL)	37.0 ± 5.9	30.9 ± 3.4	0.05

TABLE 3	CREATININE CONCENTRATIONS IN URINE AND ITS CLEARANCE IN SAMPLES WITH AND WITHOUT DEPROTEINIZATION. SERUM CREATININE CONCENTRATIONS AND CLEARANCES IN SAMPLES WITH AND WITHOUT DEPROTEINIZATION		
	Conventional samples	Deproteinization Samples	*p*
Urine creatinine (mg/dL) (N = 18)	84.38 ± 22.02	79.82 ± 23.26	0.55
Creatinine clearance (ml/min) (N = 18)	1.20 ± 0.17	1.13 ± 0.21	0.37
Serum creatinine (mg/dL) (N = 24)	0.54 ± 0.08	0.79 ± 0.63	0.07
Creatinine clearance (ml/min) (N = 24)	1.20 ± 0.17	1.24 ± 1.22	0.87

the conventional and deproteinized sera samples analyzed by ELISA (r = 0.926, *p* = 0.074). In comparing the results of the two units, there was no significant correlation and no significant difference was found either between the conventional samples (r = 0.930, *p* = 0.70) or among the deproteinized samples (r = 0.610, *p* = 0.390).

To standardize proteinuria measurements, we calculated the 24-hour PT and the rP/C from all urine samples collected. The mean 24-hour PT 24 value was 11.92 ± 6.58 mg/dL and the rP/C mean value was 1.26 ± 0.72. There was a strong correlation between the 24-hour Pt and rP/C values when assessed by the Pearson's correlation (r = 0.94, *p* < 0.001) (Figure 2A). The Bland - Altman graphs showed that rP/C is systematic and it linearly overestimates the 24-hour PT (Figure 2B). Moreover, by using the regression model, we found an equation that allowed estimating the 24-hour PT using the rP/C; 24-hour PT = (8.6113 + rP/C) + 1.0869.

DISCUSSION

Different methods are employed in the collection and processing of fluids from experimental animals; and technological refinement in scientific research has generated a significant reduction in the number of animals used, also reducing final result variability.[2] The present study showed that factors such as 24-hour urine collection, the period of fasting prior to blood collection and sample preparation procedures are important and relevant in the assessment of kidney function and should be standardized in order to obtain reliable results.

GFR is the most widely used measure for qualitative and quantitative evaluation of the kidneys' excretory capacity, and it is more accurately obtained in clinical practice from creatinine clearance, based on urine sample collected during 24 hours.[20] The Urine Collection experiment showed that the mean volume of urine from rats in the G24 group was 10.9 ± 2.2 ml, consistent with values reported by other authors, which vary between 8 and 11 ml.[21-23] Urine output volume is influenced by changes in water intake due to changes in temperature, humidity, health status and sexual activity.[22,24] We noticed that in the G12 group, urine volume was reduced by half because of a reduction in the time the animals remained in the metabolic cages and water intake reduction in relation to group G24. Even though nocturnal, about

Figure 1. Analysis of the deproteinization experiment. A: Correlation between the conventional urine creatinine and the deproteinized urine creatinine. Curve and regression 95% confidence interval, N = 18; B: Bland-Altman graph, to determine the degree of agreement between conventional urine creatinine and the deproteinized urine creatinine, N = 18; C: Correlation between the conventional serum creatinine and the creatinine in the deproteinized serum. Curve and 95% regression confidence interval, N = 24; D: Bland-Altman graph to establish the agreement between the creatinine in the conventional serum and the creatinine in the deproteinized serum, N = 24.
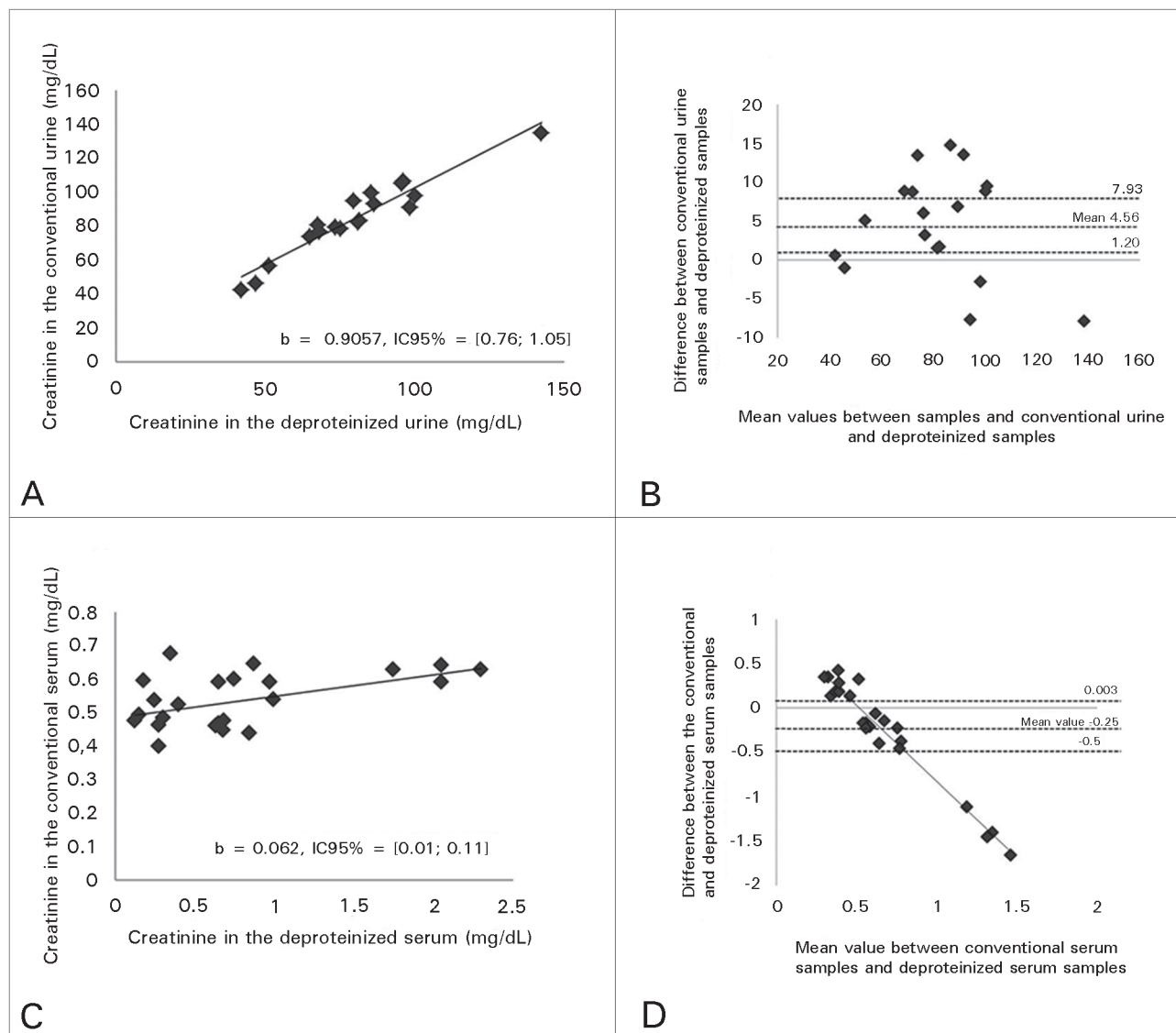

TABLE 4	SERUM CREATININE LEVELS FROM GROUP IR24, FROM CONVENTIONAL AND DEPROTEINIZED SAMPLES, ANALYZED BY AN AUTOMATIC ANALYZER THROUGH THE SPECTROPHOTOMETRIC METHOD AND THE ELISA READER		
	Creatinine in the convention samples (mg/dL) N = 4	Creatinine in deproteinized samples (mg/dL) N = 4	*p*
Spectrophotometry	2.03 ± 0.42	2.29 ± 0.36	0.38
ELISA	1.83 ± 0.34	2.42 ± 0.37	0.06
p	0.49	0.63	

57% of these rodents' urine volumes may have been secreted during the day. Similar results were found for the Control Group in a study using male Wistar rats, and part of the diuresis was reported during the day (4.6 ± 0.5 ml).[25] The mean feed intake and water intake for rats are, respectively, 5 g and 10 ml per 100 g of body weight, and there is strong correlation saying that as more food is consumed more water is ingested.[24] The feed intake of the groups observed for 24 hours and 12 hours was similar to that reported previously, including keeping the pattern of increased intake during the night.[22,24,25]

Figure 2. Experiment analysis methods - 24-hour proteinúria estimate. A: Correlation between the 24-hour proteinúria and the protein/creatinine ratio. Curve and 95% regression confidence interval, N = 59; B: Bland-Altman graph used to establish the degree of agreement between 24-hour proteinúria and the protein/creatinine ratio, N = 59.
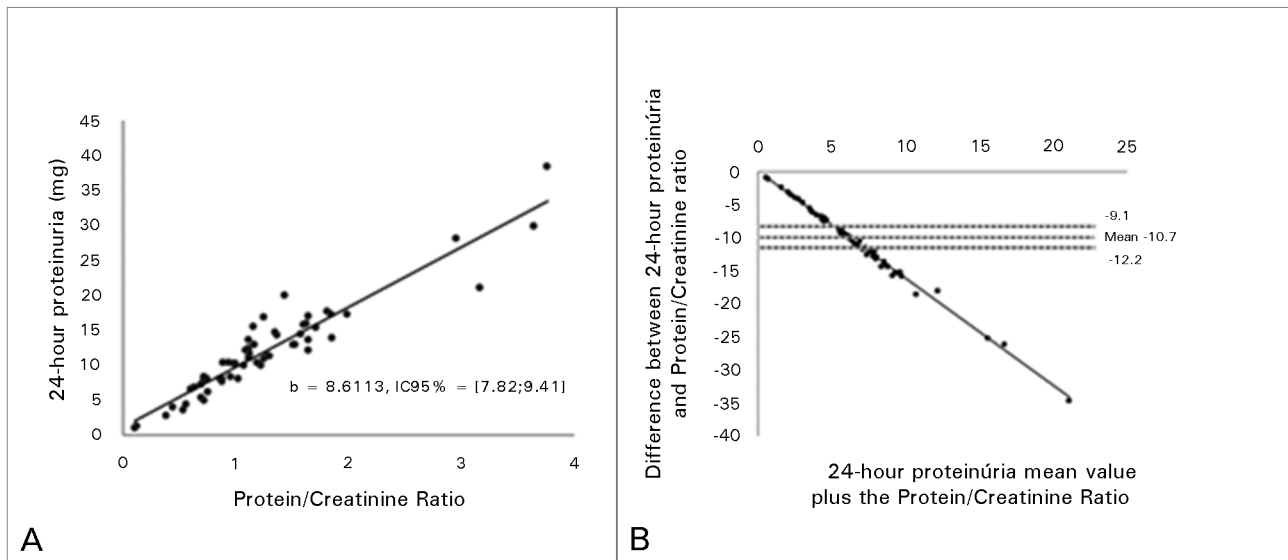
Creatinine clearance is measured using urine flow and the creatinine values measured in the urine and plasma.[9] The creatinine concentration in the urine sample collected over a period of 12 hours was similar to values found in the 24-hour urine Group. Results from Brazilian colonies reported 24-hour urine values ranging around 67.2 mg/dL in young animals to 75 mg/dL in adult rats, similar to those found in this study.[11,17] Even with similar urinary creatinine concentration, the animals of the G24 Group had higher creatinine clearance when compared to those in the G12, a consequence of the lower urinary volume obtained, even after correction, with duplication of the collection time, and lower serum creatinine. However, the difference found between the serum creatinine from Groups G24 and G12 is no different from the biological standpoint, since the mean values are close to those described as normal. Previous studies have found values of 0.83 ± 0.07 ml/min and 0.74 ± 0.01 ml/min of creatinine clearance in adult rats, leading us to assume that such variations may be due to environmental factors, stress, genetic factors or even methodological variables.[17,21]

The 24-hour PT value from both groups (G24 and G12) are similar to those found in the literature.[26,27] Furthermore, since there was no rP/C difference from one group to another, it is suggested that the values found in the 12-hour urine can be used to assess proteinuria.

By reducing the intake of salt, proteins and carbohydrates contained in feed, there are changes in the physiological mechanisms that regulate fluid homeostasis, which leads the animals to ingest smaller volumes of liquid.[28] The animals from the JE12 group were not fed for an extended period of 12 hours, which resulted in a decrease of approximately 70% in fluid intake, with respect to Group G12. Nonetheless, there was a significant increase in diuresis among the fasted animals; however, without characterizing abnormality, since diuresis (7.9 ± 2.6 ml) and water intake (5.8 ± 2.6 ml) are similar to the values described.[11,14,15,21-23]

The number of hours in fasting is defined according to the test to be carried out and this need stems from the fact that food can momentarily change blood composition, inducing a possible error in reading the samples.[29] Two tests widely used in monitoring kidney function are the measurements of serum creatinine and urea, which vary in function of physiological factors, such as protein intake and its catabolism.[30] Although fasting is clearly not recommended for measuring serum creatinine, higher values were found in the group without fasting, but still within the normal range. This difference is not representative of kidney dysfunction detection, considering that in the literature there are similar values for animals in control groups. In standardization experiments, the serum creatinine reference value in the serum of normal animals was 0.50 ± 0.07 mg/dL - adult male

rats maintained in fasting for 12 to 15 hours.[11,12] In other studies, the normal animals had mean values of 0.60 ± 0.10 mg/dL[31] and 0.60 ± 0.02 mg/dL[32] of serum creatinine, demonstrating that there may be small intra-species variations and variations between colonies as well.

Fasting did not influence urea values. Values close to those found in this study have been described in the literature, from studies that submitted male, adult and healthy rats to 12 hours of fasting.[12,32] In the literature there are also higher values for animals in these same health status, but not submitted to fasting.[31]

Serum creatinine values were also not significantly altered by fasting; however, urine creatinine was lower, which also altered creatinine clearance values. The hypothesis is that this feed present in the cages of the G12 group may have contaminated the urine collectors and have been read as creatinine. It had already been reported that food particles are poured while the animals remain in metabolic cages.[33] It has also been reported the supply of feed in powder form, in an attempt to reduce collectors' contamination with food particles.[25] The lack of feed during 12 hours nightly for the JE12 group may be the cause of the decrease in creatinine values in urine. Nevertheless, these values did not characterize major changes in kidney function from the biological point of view, and were similar to values found in the literature.[11,17] The same can be said for the 24-hour PT values.

The serum creatinine test is advantageous; however, serum creatinine levels do not increase significantly until kidney function is considerably compromised.[34,35] Furthermore, there are some substances that may be erroneously read as creatinine (chromogenic bodies); some of these are normally present in blood or urine, or are found only in sickness.[36]

Several approaches have been presented in an attempt to reduce the interference suffered by body fluids.[37] In this study the authors assessed protein interference in the deproteinization experiment. The conventional urine samples showed higher mean creatinine vis-à-vis the samples of deproteinized urine. Although there was no significant difference between the mean values, we found a reduction of less than 5 mg/dL of creatinine in conventional urine when compared with the deproteinized urine,

characterizing the possible presence of interfering protein substances in the conventional urine. Moreover, the correlation and lack of bias between the conventional urine and the deproteinized one led us to suppose that the deproteinization procedure eliminated such interferences and enabled more reliable measurement of creatinine in urine. However, there was no difference from the biological point of view, showing that such interference behaves in a systematic manner for all animals.

In contrast, the results found in the serum samples were contrary to those from the urine samples. We expected the deproteinization to eliminate interfering substances, thus lower deproteinized serum creatinine values; however, this did not happen. There was no significant difference between the samples, and the correlation was positive; but there was an increase in mean creatinine levels from conventional samples when compared to deproteinized samples. This increase was not linear for all samples alone, the standard deviation was several times higher, showing that the deproteinization procedure behaved differently for each sample. The difference between conventional serum and deproteinized serum was positive for some samples and negative for others, indicating that the deproteinization procedure was not efficient, not producing comparable results. There was evidence of linear and systematic bias in the comparison between conventional serum creatinine and the deproteinized serum, revealing the inefficiency of the sample deproteinization. In a study that also used the deproteinization method, the authors found lower serum creatinine values from normal adult rats (0.36 ± 0.02 mg/dL).[17] As to the mean creatinine clearance values, there was no significant difference between the deproteinized and the conventional samples, probably due to lack of linearity of deproteinized samples, the standard deviation was much higher for this group.

After verifying that the deproteinization in conventional serum was not linear, we decided to apply the method to the serum of animals with AKI, which would knowingly have elevated serum creatinine, considering that, in disease conditions, creatinine concentrations may be impacted by interfering agents.[36]

Two reading devices were used: the spectrophotometric automatic analyzer and the

ELISA reader - theoretically with superior reading efficiency. However, the values read were not different, leading us to believe that there would be no need to use more accurate equipment. Creatinine values found in samples from animals with AKI, analyzed by the two devices, were in agreement with studies which also used the AKI model.[14,38] Thus, the deproteinization of serum samples did not bring significant information to the interpretation of results vis-à-vis the creatinine concentration.

Studies in humans try to enhance the means of urine collection, proposing a single sample collection and estimating 24-hour PT by means of the rP/C. In these studies and after many years of clinical application, the values are used without the need for correction.[19,39] In animals,[27,40] as found in this study, the relationship between the values are not the same. Employing the linear regression model, the mathematical relationship between two variables was found, thus creating a practical way to estimate these animals' 24-hour PT from the results of a urine sample. This equation will provide greater animal welfare because it will enable a reduction in the time the animals have to stay in metabolic cages. Enabling, in some cases, spot urine collection at a specific time of day without having to feed the animals, avoiding contamination by the feed.

One limitation of this study was the small sample unit: 4-6 animals per group. There is no consensus on the number of animals for standardization of assessment techniques, the sample size was based on mathematical relationships, determining the number of animals required to reach significance at a $p < 0.05$. Another limitation relates to the existing divergences among studies concerning the deproteinization method.

Conclusions

From the presented data we concluded that, for rats from the Center of Reproduction Biology colony:

Kidney function assessment should be performed with the 24-hour urine collection.

Fasting proved unnecessary, as was the deproteinization procedure.

Spectrophotometry reading proved to be viable.

Based on the rP/C values, we proposed a practical equation to estimate 24-hour PT in this group of animals, enabling the spot collection of urine and avoiding contamination by feed.

The kidney function assessment methodology definition will enable the production of better quality scientific evidence, besides enhancing the number and the well-being of the animals involved in the trial.

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Errata

The paper: "Standardizing kidney function evaluation in Wistar rats (*Rattus norvegicus*) from the vivarium of the Federal University of Juiz de Fora", published in the April 2014 issue of the Brazilian Journal of Nephrology [J Bras Nefrol. 2014; 36: 139-49] had Figures 1 and 2 changed.