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TESIS DOCTORAL

CONSECUENCIAS DE LA UNINOFRECTOMÍA SOBRE LA FUNCIÓN CARDIOVASCULAR Y RENAL EN RATAS. UTILIDAD DE LAS AMINOPEPTIDASAS URINARIAS COMO MARCADORES DE DISFUNCIÓN RENAL

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Andrés Quesada Miñarro
Jaén 2014

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CERTIFICA:

Que los trabajos efectuados en la elaboración de la Tesis Doctoral titulada: **"*Consecuencias de la uninefrectomía sobre la función cardiovascular y renal en ratas. Utilidad de las aminopeptidasas urinarias como marcadores de disfunción renal",*** presentada po***r*** **Andrés Quesada Miñarro**, han sido realizados bajo mi supervisión y dirección, reuniendo las condiciones académicas necesarias para su presentación para optar al grado de Doctor.

Y para que conste donde proceda, firmo la presente en

Jaén, a 28 de Enero de 2014

Fdo. Rosemary Wangensteen Fuentes

D. JOSÉ FÉLIX VARGAS PALOMARES, DOCTOR EN MEDICINA Y CATEDRÁTICO DEL DEPARTAMENTO DE FISIOLOGÍA DE LA FACULTAD DE MEDICINA DE LA UNIVERSIDAD DE GRANADA

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Que los trabajos efectuados en la elaboración de la Tesis Doctoral titulada: **"*Consecuencias de la uninefrectomía sobre la función cardiovascular y renal en ratas. Utilidad de las aminopeptidasas urinarias como marcadores de disfunción renal*"**, presentada por **Andrés Quesada Miñarro**, han sido realizados bajo mi supervisión y dirección, reuniendo las condiciones académicas necesarias para su presentación para optar al grado de Doctor.

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ÍNDICE

1.INTRODUCCIÓN

La donación de riñón inter-vivos cuenta con altas tasas de supervivencia para los receptores y hasta el momento se considera que existe un riesgo bajo para los donantes (Sommeder y cols., 2004). Sin embargo, las perspectivas iniciales de la donación inter-vivos se vieron truncadas en parte, debido a la publicación en la década de los 80 de algunos estudios experimentales en los que se observó la existencia de hiperfiltración después de la uninefrectomía, y se empezó a pensar que la donación de riñon inter-vivos podría provocar proteinuria, hipertensión y glomeruloesclerosis (Oppenheimer Salinas, 2010).

Ciertamente, es posible que el impacto de la donación sobre la presión arterial y la función renal haya sido subestimado debido a las dificultades que presenta la valoración adecuada de estos parámetros. Además, no se ha analizado suficientemente el efecto de diversos factores que pueden conllevar un riesgo potencial para el donante. A pesar de lo anteriormente expuesto, se ha producido un gran aumento de las donaciones inter-vivos en los últimos años (Ommen y cols., 2006) debido, por una parte, a la limitada disponibilidad de órganos de donantes fallecidos y, por otra parte, a la creciente demanda de trasplantes. Como resultado, miles de individuos sanos están siendo uninefrectomizados cada año. Este hecho ha generado ciertas interrogantes en algunos autores sobre las consecuencias a largo plazo de la uninefrectomía (Ommen y cols., 2006; Delmonico, 2005).

Los datos existentes sobre la relación entre la disminución del número de nefronas y el riesgo de aumento en la presión arterial y/o de proteinuria no son muy consistentes. Se ha publicado que la disminución inicial de la función renal se ve compensada en parte por un cambio en la tasa de filtración glomerular media del 70% al 75% del valor previo a la uninefrectomía (Vincenti y cols., 1983; Edgren y cols., 1976). Algunos investigadores han encontrado un aumento de la presión arterial posterior a la donación del riñon (Hakim y cols.,1984; Torres y cols., 1987), y los estudios que han comparado los donantes con sus propios hermanos o con otros donantes potenciales han obtenido resultados contradictorios sobre el impacto de la donación en la presión arterial, la función renal y la proteinuria (Hakim y cols., 1984; Bock y cols., 1992; Chavers y cols., 1985; Willians y cols., 1986; Baudoin y cols., 1993). Hay que tener en cuenta la dificultad que presenta disociar los efectos del proceso de envejecimiento de la influencia de la uninefrectomía, porque la edad influye tanto en la función renal como

en los niveles de presión arterial del individuo. Así, en estudios de poblaciones clasificadas por edad y sexo, algunos autores (Bock y cols., 1992; Willians y cols., 1986; Baudoin y cols., 1993) encontraron diferencias entre la presión arterial de los donantes de riñón y la población general. Además, las ratas sometidas a uninefrectomía con tres semanas de edad desarrollaron hipertensión y disminución de la tasa de filtración glomerular entre las seis y ocho semanas de vida en comparación con los controles (Carlstrom y cols., 2007). Zheng y cols. (2011) encontraron que la uninefrectomía en ratones provocaba una nefropatía diabética más avanzada en los ratones diabéticos, pero casi no tenia efectos en los ratones no diabéticos.

Los estudios que se han realizado sobre los donantes humanos de riñón han estado bastante limitados, ya que, los test invasivos se llevan a cabo solamente en el caso de que existan alteraciones clínicas, siendo poco factible reclutar donantes vivos para realizar un seguimiento a largo plazo de los parámetros de morfología y de función renal.

Por tanto, se hace patente la necesidad de marcadores que proporcionen información sobre el grado de disfunción renal y que estén relacionados con las alteraciones en la morfología del riñón. Estos marcadores permitirían llevar a cabo el seguimiento de estos pacientes sin tener que recurrir a procedimientos invasivos.

Para averiguar la utilidad diagnóstica de un posible marcador de disfunción renal, es necesario analizar su sensibilidad y especificidad tanto en modelos experimentales de daño renal agudo como en modelos de enfermedad renal crónica.

El daño renal agudo es un problema clínico habitual que puede definirse como un aumento brusco de la concentración plasmática de creatinina observable en un período inferior a 48 horas, y que tiene su origen en un daño que provoca un cambio funcional o estructural en el riñón. La causa principal de daño renal agudo es la apoptosis o necrosis aguda de las células de los túbulos renales. Para clasificar el grado de daño renal se suele utilizar la escala RIFLE, acrónimo de las palabras inglesas “risk” (riesgo), “injury” (daño), “failure” (fallo), “loss” (pérdida) y “end-stage renal disease” (enfermedad renal en estadío terminal). El criterio en el que se basa esta escala para clasificar la disfunción renal de los pacientes es la concentración de creatinina plasmática (Molitoris y cols., 2007).

Sin embargo, se ha publicado que los marcadores de daño renal agudo utilizados tradicionalmente, como son la urea y la creatinina plasmáticas, carecen de sensibilidad y especificidad, y no diferencian adecuadamente entre los diferentes grados de daño renal

agudo (Vaidya y cols., 2008). La creatinina se va acumulando con el paso del tiempo, por lo que sus niveles en sangre no se corresponden exactamente con el estado fisiopatológico en que se encuentra en el riñón y, por otra parte, los cambios en la concentración plasmática de creatinina sólo son observables cuando los riñones han perdido un 50 % de su capacidad funcional (Bonventre y cols., 2010; Devarajan, 2007).

La detección precoz del daño renal continúa siendo un reto en la actualidad, tanto en la práctica clínica como en la investigación preclínica, y hay una urgente necesidad de encontrar mejores biomarcadores que sean capaces de detectar de manera temprana el daño, que sean capaces de predecir la intensidad del daño, y que proporcionen información sobre la seguridad de los fármacos durante el desarrollo de los ensayos clínicos.

Las enzimas liberadas desde las células tubulares dañadas y excretadas hacia la orina son los biomarcadores más prometedores para realizar el diagnóstico precoz del daño renal agudo. La determinación de estas enzimas puede proveer información detallada sobre la naturaleza, tamaño y lugar del daño en las células tubulares y sobre su posible disfunción o necrosis (Lisowska-Myjak, 2010). Una de estas enzimas, alanil aminopeptidasa (AlaAp) (EC 3.4.11.2), es una enzima del borde en cepillo que ya fue propuesta por Peters y cols. en 1972 como marcador urinario de la enfermedad renal, existiendo en la actualidad un ensayo fotométrico automatizado para su determinación (Holdt y cols., 2008).

AlaAp, junto con glutamil aminopeptidasa (GluAp) (EC 3.4.11.7), cistinil aminopeptidasa (CysAp) (3.4.11.3) y aspartil aminopeptidasa (AspAp) (EC 3.4.11.21) se encuentran formando parte de las células del túbulo renal (Kenny y Maroux, 1982; Song y cols., 1994; Albiston y cols., 2011), donde ejercen su función aminopeptidásica en el metabolismo de la angiotensina II, péptido que aparece elevado en los trastornos renales. En nuestro laboratorio, hemos determinado la actividad de estas cuatro aminopeptidasas como índices del daño renal en ratas hipertiroideas y en ratas sometidas a una alta ingesta de sal (Pérez-Abud y cols., 2011).

En esta tesis se ha investigado la utilidad de la determinación fluorimétrica de las actividades enzimáticas en orina de AlaAp, GluAp, CysAp y AspAp como biomarcadores de la disfunción renal inducida por cisplatino a la dosis de 7 mg/kg en ratas.

El cisplatino es un fármaco antineoplásico que tiene un efecto nefrotóxico directo sobre el túbulo proximal tanto en animales como en seres humanos (Winston y

Safirstein, 1985; Safirstein y cols., 1987). En las ratas tratadas con una sola dosis de cisplatino de 7 mg/kg se puede observar degeneración tubular y necrosis (Yu y cols., 2010), junto con un aumento en la excreción de N-acetil-β-D-glucosaminidasa (NAG) (Kawai y cols., 2009), una enzima tubular renal (Bazzi y cols., 2002) y de glucosuria (Kawai y cols., 2009), que alcanza un máximo al tercer día de la inyección de cisplatino. Las concentraciones de creatinina y de urea en plasma alcanzan su nivel máximo a los 7 días (Kawai y cols., 2009) y se puede observar regeneración y dilatación del epitelio tubular a los 8 días del tratamiento (Yu y cols., 2010). Desde el dia 14 al 56 (después de la inyección de cisplatino), estos animales desarrollan fibrosis tubulointersticial, la cual no va acompañada de diferencias en la excreción urinaria de NAG o de glucosa, aunque sí se observa un incremento significativo de la creatinina y de la urea en plasma a los 56 días (Kawai y cols., 2009).

2.OBJETIVOS

En base a los antecedentes anteriormente expuestos, nos planteamos los siguientes objetivos:

1) Examinar las consecuencias de la uninefrectomía, como un modelo de nefrectomía de donante, sobre la presión arterial, el manejo de sodio renal, la sensibilidad a la sal, el estrés oxidativo y el daño renal a lo largo de 18 meses, tanto en ratas macho como en ratas hembra.

2) Determinar el papel de las aminopeptidasas urinarias como biomarcadores tempranos del daño renal agudo en un modelo animal, evaluando su excreción urinaria durante los 3 días posteriores a la inyección de cisplatino.

3) Analizar el valor de las aminopeptidasas urinarias como biomarcadores predictores del grado de disfunción renal, correlacionando la cantidad excretada del marcador en las primeras 24 horas con dos parámetros de función renal (creatinina plasmática y aclaramiento de creatinina), y dos parámetros de daño estructural (hipertrofia renal y fibrosis intersticial).

4) Establecer la sensibilidad y especificidad de cada marcador a la hora de distinguir a los animales tratados con cisplatino de los animales control.

3. RESUMEN GLOBAL DE LOS RESULTADOS

3.1. Resultados sobre las consecuencias de la uninefrectomía en ratas

3.1.1. Variables morfológicas

No se encontraron diferencias en cuanto al peso corporal entre los grupos control y los grupos de ratas uninefrectomizadas (UNX) ni a los 6 ni a los 12 meses después de la intervención. Sin embargo, encontramos una reducción significativa de los pesos corporales en ambos sexos a los 18 meses.

En cuanto al peso del riñón y del corazón al final del experimento, observamos que el peso de estos dos órganos en las ratas hembra era inferior al peso de las ratas macho y, además, encontramos que la *ratio* peso renal/peso corporal y la *ratio* peso cardíaco/peso corporal estaban significativamente aumentadas en los grupos con UNX respecto a sus controles.

La hipertrofia renal compensatoria que se desarrolla en este modelo de UNX resultó ser significativamente superior ($P<0.05$) en las ratas macho que en las ratas hembra.

La *ratio* peso cardíaco/peso corporal y la *ratio* peso ventricular/peso corporal eran mayores en las ratas hembra que en las ratas macho y las ratas hembra UNX mostraban valores superiores en la *ratio* peso ventricular/peso corporal que los de su respectivo grupo control.

3.1.2. Presión arterial

Se encontraron valores de presión arterial más elevados en las ratas macho UNX que en el grupo control 6 meses después de la uninefrectomía. Sin embargo, en este punto no se encontraron diferencias entre las ratas hembra y el grupo control.

Ttranscurridos 12 y 18 meses de la intervención, los valores de presión arterial en las ratas UNX de ambos sexos, fueron superiores a los obtenidos en sus respectivos grupos control. Paralelamente, observamos una diferencia entre sexos en los grupos UNX, de manera que a los 18 meses las ratas macho mostraron valores más altos de presión arterial que las ratas hembra.

3.1.3. Manejo renal de sodio

No se observaron diferencias en los niveles basales de excreción de agua y sodio de las ratas UNX con respecto a los controles en ninguno de los dos sexos, ni tampoco se observaron diferencias en el manejo renal de sodio en ningún punto del experimento.

Por otro lado, sí que pudimos observar una diferencia entre sexos, en los niveles de diuresis y natriuresis, ya que éstas aparecían más elevadas a los 6, 12 y 18 meses de la uninefrectomía en las ratas hembra.

Mediante el aumento en la ingesta de sal (1% NaCl en el agua de bebida) durante dos semanas a los 6, 12, y 18 meses, se comprobó que la relación presión-natriuresis se encontraba desplazada a la derecha a los 6 meses en el grupo de ratas macho UNX, y en los 12 y 18 meses siguientes también aparecía desplazada a la derecha en el grupo de hembras sometidas a UNX con respecto a su grupo control.

3.1.4. Variables renales y plasmáticas al final del experimento

Las concentraciones plasmáticas de urea y creatinina determinadas al final del experimento estaban más elevadas en los dos grupos de ratas UNX, mientras que la concentración de proteínas en plasma resultó ser ligeramente mas baja en ambos grupos UNX, aunque sin llegar a ser estadísticamente significativa. Por otro lado, los niveles de sodio y potasio plasmáticos fueron similares en todos los grupos estudiados.

El aclaramiento de creatinina estaba disminuido en los dos grupos de ratas UNX con respecto al de los controles al final del experimento. Sin embargo, tras someter a las ratas a la sobrecarga salina de dos semanas, los valores de aclaramiento de creatinina en el grupo de machos UNX y controles, no experimentaron cambios significativos respecto al valor basal, pero sí encontramos un incremento respecto a los valores basales en los grupos de ratas hembra.

El colesterol total medido en plasma, aparecía disminuído en las ratas hembra UNX con respecto a los machos UNX, mientras que los valores de HDL y LDL eran inferiores en el grupo control de ratas hembra con respecto al grupo control de ratas macho.

3.1.5. Alteraciones fisiopatológicas producidas por el aumento en la ingesta salina

Como resultado del aumento en la ingesta salina durante dos semanas, se encontró que la presión arterial en los dos grupos de ratas UNX aumentaba tanto a los 6 como a los 12 y 18 meses del inicio del experimento. Sin embargo, la ingesta salina no produjo ningún aumento en los niveles de presión arterial de las ratas control macho, aunque sí se observó un aumento de la presión arterial en las ratas control hembra a los 18 meses de la cirugía.

A los 6 meses del inicio del estudio, no observamos ninguna alteración en los valores de proteinuria en ningún grupo, pero, conforme avanzaba el experimento, y de forma general, a los 12 y 18 meses los valores de proteinuria en todos los grupos se encontraron aumentados al realizar un análisis pareado de los datos con respecto a las muestras obtenidas a los 6 meses del estudio y, aunque los valores de proteinuria determinados a los 18 meses eran muy elevados en todos los grupos, el incremento fue superior en los grupos de ratas UNX que en los grupos de ratas control. Pudimos observar también que, en el grupo de ratas macho UNX, el valor de la proteinuria estaba aumentado significativamente con respecto a sus controles a los 12 meses del inicio del estudio.

Los valores de N-acetil-β-*D*-glucosaminidasa (NAG) también reflejaron alteraciones cuando los distintos grupos fueron sometidos al aumento en la ingesta salina. Por una parte, los grupos de ratas UNX experimentaron un aumento en la excreción de NAG desde la toma correspondiente a los 6 meses, incrementándose la pendiente de la recta realizada entre los valores obtenidos antes y después de la ingesta salina en estos grupos en los sucesivos meses. Por otra parte, en el grupo de ratas macho control, observamos que comenzaban a aumentar los valores de NAG a los 12 meses como consecuencia de la ingesta salina, y a los 18 meses la ingesta de sal provocó un incremento en la excreción de NAG tanto en el grupo de ratas macho como en el grupo de ratas hembra control.

Los niveles de 8-isoprostano más elevados los encontramos en los grupos de ratas hembra a lo largo de todo el experimento. Concretamente, el grupo de ratas hembra UNX en la toma correspondiente a los 18 meses, resultó ser el que mayores valores proporcionó de todos los grupos estudiados. Los valores de 8-isoprostano mostraron un

marcado aumento tanto en la toma correspondiente a los 12 meses como en la toma de los 18 meses en ambos grupos de ratas UNX, mientras que sólo observamos un ligero incremento en los grupos control y tan sólo en el mes 18.

3.2.6. Variables de inflamación renal y de estrés oxidativo

Se cuantificaron diversas variables relacionadas con fenómenos inflamatorios y/o oxidativos en el tejido renal de los diferentes grupos de animales al final del experimento. Estas variables fueron: el factor de crecimiento transformante-β (TGF-β), el colágeno tipo III, la concentración de nitratos y nitritos, y los niveles de 8-isoprostano. Sin embargo, en ninguno de los parámetros analizados pudimos observar diferencias significativas entre los grupos.

3.1.7. Resultados del estudio histopatológico y de la cuantificación de la fibrosis intersticial

Se encontraron algunos cambios morfológicos estructurales en los grupos de ratas control sacrificadas a los 18 meses, incluyendo un ligero aumento de la matriz mesangial con esclerosis glomerular y sinequias, aspecto quístico de algunos glomérulos con un cierto grado de atrofia, así como la presencia atrofia tubular.

La cantidad de cilindros tubulares, los enquistamientos glomerulares y la hipercelularidad glomerular estaban aumentados significativamente en las ratas macho control en comparación con las ratas control hembra a los 18 meses de la cirugía.

En general, las lesiones a nivel glomerular y túbulo-intersticial, y las lesiones vasculares fueron significativamente más severas en las ratas UNX macho que en las ratas UNX hembra y que en las ratas macho control a los 18 meses de edad. El examen histopatológico de los cortes renales de las ratas UNX macho, mostró alteraciones importantes, como son: abundantes cilindros tubulares, marcada dilatación tubular, atrofia tubular incipiente e infiltrado inflamatorio focal.

En comparación con los otros grupos, las ratas macho UNX mostraron esclerosis glomerular más intensa, aumento de la matriz mesangial, atrofia tubular, y presencia de cilindros tubulares, con engrosamiento de la membrana basal tanto en estructuras tubulares como capsulare, así como infiltrado inflamatorio crónico y arteriopatía hialina en mayor grado.

El grado de fibrosis intersticial que obtuvimos en los grupos UNX fue superior al que observamos en sus respectivos grupos control, y más aún en el grupo de animales UNX macho que en cualquier otro grupo del estudio. Observamos que el porcentaje de tejido conectivo intersticial en el grupo de animales macho control, era más alto que el 5-8%, que es el porcentaje más habitual en ratas jóvenes de 2 a 4 meses, lo que evidencia el aumento del grado de fibrosis renal que se produce con la edad.

3.2. Resultados sobre las aminopeptidasas urinarias como marcadores tempranos y predictores de la disfunción renal.

3.2.1. Excreción de los distintos marcadores urinarios en los días posteriores al tratamiento con cisplatino

Las actividades glutamil aminopeptidasa (GluAp), alanil aminopeptidasa (AlaAp), cistinil aminopeptidasa (CysAp) y aspartil aminopeptidasa (AspAp) estaban aumentadas significativamente en las muestras urinarias recogidas en los días 1, 2 y 3 posteriores al tratamiento con cisplatino a la dosis de 7 mg/kg, mientras que sólo se encontró un ligero aumento, aunque significativo estadísticamente, en la actividad CysAp en la muestra correspondiente al día 2 del tratamiento con cisplatino a la dosis de 3.5 mg/kg.

Los valores de albuminuria se encontraron aumentados durante los tres días de recogida, mientras que otros marcadores estudiados como son la proteinuria, la NAG y la lipocalina asociada a la gelatinasa de neutrófilos (NGAL), se elevaron solamente en la muestra recogida al tercer día del tratamiento con la dosis de 7 mg/kg de cisplatino.

En el grupo tratado con una dosis subtóxica (3.5mg/kg) la excreción de proteinuria y NGAL estaban aumentadas el día 3 del experimento.

3.2.2. Efectos del tratamiento con cisplatino sobre la función renal.

A las dos semanas de la inyección con cisplatino, observamos una disminución del aclaramiento de creatinina, acompañado de un aumento de los niveles de creatinina en plasma y de la *ratio* peso renal/peso corporal en el grupo de animales tratados con 7mg/kg de cisplatino.

En ese mismo punto, en el grupo tratado con 3.5 mg/kg no se encontraron diferencias significativas respecto al grupo control.

3.2.3. Alteraciones histopatológicas provocadas por el cisplatino

No se encontraron lesiones renales a nivel histológico en el grupo control, ni tampoco se observaron alteraciones túbulo-intersticiales, vasculares o del parénquima renal.

Sin embargo, en los grupos tratados con cisplatino a ambas dosis observamos un incremento en el proceso de displasia nuclear de las células renales y una incipiente necrosis tubular.

Además, en los dos grupos experimentales tratados con cisplatino, existía una marcada dilatación de los túbulos proximales de la zona cortico-medular en casi todo el epitelio debido a la descamación del epitelio tubular, que indujo a una ausencia total de microvellosidades y pérdida del borde en cepillo en el grupo tratado con la dosis máxima de cisplatino. También encontramos una ligera atrofia tubular y células apoptóticas en el lumen tubular, presentes en ambos grupos tratados con cisplatino.

Se encontraron diferencias estadísticamente significativas entre ambos grupos de cisplatino en el análisis de las variables morfológicas, lo que indica una relación dosis-respuesta.

El tratamiento con cisplatino aumentó significativamente el área de fibrosis en el intersticio tubular en ambos grupos experimentales. El área de fibrosis era mucho mayor en la unión cortico-medular del riñón.

3.2.4. Valor predictivo de los diferentes marcadores urinarios analizados sobre las alteraciones estructurales y funcionales del riñón.

Obtuvimos altos coeficientes de correlación entre la actividad de las cuatro aminopeptidasas analizadas en orina, trascurridas las 24 primeras horas del tratamiento, con respecto a la *ratio* pesor renal/peso corporal.

También se observaron correlaciones entre la actividad de AlaAp y CysAp y el aclaramiento de creatinina, así como entre la actividad urinaria de AlaAp y la concentración de creatinina en plasma.

Por otra parte, no pudimos encontrar correlación significativa entre los marcadores analizados en la muestra recogida a las 24 horas del tratamiento y el porcentaje de fibrosis al final del experimento.

3.2.5. Sensibilidad y especificidad de los diferentes marcadores

La actividad de AlaAp en orina fue la que mayor porcentaje de área bajo la curva (AUC-ROC) obtuvo de todos los marcadores estudiados, transcurridas las primeras 24 horas del tratamiento, es decir, la determinación de la actividad de AlaAp fue la que mayor sensibilidad y especificidad mostró para poder diferenciar entre animales que recibieron cisplatino o salino.

El área bajo la curva que obtuvimos para el caso de GluAp, CysAp y AspAp fue mayor a 0.5 en los cuatro casos, al igual que la de otros marcadores como proteinuria y albuminuria, por lo que las cuatro enzimas urinarias pueden ser consideradas como biomarcadores de disfunción renal en este modelo animal.

4.CONCLUSIONES

1. La uninefrectomía en ratas jóvenes macho y hembra produce a largo plazo un aumento de presión arterial y se asocia a signos plasmáticos, urinarios y morfológicos indicativos de lesión renal.
2. La uninefrectomía aumenta la sensibilidad a la sal que se manifiesta por un aumento en la presión arterial, aumento del estrés oxidativo y signos de daño renal en respuesta al aumento de ingesta salina.
3. Las ratas macho son más sensibles a los efectos a largo plazo de la uninefrectomía, observándose un aumento precoz de la presión arterial y del daño renal respecto a las ratas hembra.
4. La determinación en orina de la actividad alanil, glutamil, cistinil y aspartil aminopeptidasa permite detectar de manera precoz y predecir el daño renal provocado por la administración de cisplatino.
5. Las aminopeptidasas muestran mayor sensibilidad y especificidad que el resto de marcadores analizados para diferenciar a los animales tratados con cisplatino de los animales control 24 horas después del tratamiento.

5.PUBLICACIONES QUE CONSTITUYEN LA TESIS

Long-Term Consequences of Uninephrectomy in Male and Female Rats

Isabel Rodríguez-Gómez, Rosemary Wangensteen, Rocío Pérez-Abud, Andrés Quesada, Raimundo G. del Moral, Antonio Osuna, Francisco O'Valle, Juan de Dios Luna and Félix Vargas

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Long-Term Consequences of Uninephrectomy in Male and Female Rats

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See Editorial Commentary, pp 1383–1384

Abstract—We investigated the effects of uninephrectomy (UNX) in 6-week-old male and female rats on blood pressure (BP), renal sodium handling, salt sensitivity, oxidative stress, and renal injury over 18 months postsurgery, studying control sham-operated and UNX-operated rats at 6, 12, and 18 months postsurgery, evaluating their renal sodium handling, BP, urinary isoprostanes, *N*-acetyl-β-D-glucosaminidase, and proteinuria before and after a 2-week high-salt intake period. At 18 months, plasma variables were measured and kidney samples were taken for the analysis of renal morphology and tissue variables. BP was increased at 6 months in male UNX rats versus controls and at 12 and 18 months in both male and female UNX rats and was increased in male versus female UNX groups at 18 months. UNX did not affect water and sodium excretion under basal conditions and after the different test in male and female rats at different ages. However, the renal function curve was shifted to the right in both male and female UNX rats. High-salt intake increased BP in both UNX groups at 6, 12, and 18 months and in the female control group at 18 months, and it increased proteinuria, *N*-acetyl-β-D-glucosaminidase, and isoprostanes in both UNX groups throughout the study. Renal lesions at 18 months were more severe in male versus female UNX rats. In summary, long-term UNX increased the BP, creatinine, proteinuria, pathological signs of renal injury, and salt sensitivity. Earlier BP elevation was observed and morphological lesions were more severe in male than in female UNX rats. **(*Hypertension*. 2012;60: 1458-1463.)** ● **Online Data Supplement**

Key Words: uninephrectomy ■ renal sodium handling ■ renal injury ■ blood pressure ■ rat

Living kidney donation has achieved excellent survival rates for recipients and has been considered to pose a low risk to donors.[1] However, enthusiasm for living donation waned somewhat in the early 1980s with experimental reports of hyperfiltration after uninephrectomy (UNX), and fears were expressed that living kidney donation could cause proteinuria, hypertension, and eventual glomerulosclerosis.[2] Indeed, it is possible that the impact of donation on blood pressure (BP) and renal function may have been underestimated because of the difficulties in their measurement, and potential vulnerability factors in donors have not been well explored. Nevertheless, the limited availability of organs from deceased donors and the growing demand for transplantation have produced a marked increase in living kidney donation worldwide over recent years.[3] As a result, thousands of healthy individuals are becoming uninephric every year, generating some concerns about the long-term consequences of UNX.[3,4]

Data on the relationship between nephron loss and the risk of increased BP or proteinuria have not been consistent. It has been reported that the initial loss of renal function is, in part, compensated for producing a mean glomerular filtration rate that is 70% to 75% of the pre-UNX value.[5,6] Some researchers have found an increase in BP after kidney donation,[7,8] and studies comparing donors with siblings or potential kidney donors have shown conflicting results for the impact of donation on BP, renal function, and proteinuria.[7,9–12] It is complicated to dissociate the effects of the aging process from the influence of UNX, because both renal function and BP are influenced by age. Thus, in studies of age- and sex-matched populations, some[9,11,12] but not all[7] authors found differences in BP between kidney donors and the general population. Rats undergoing UNX at 3 weeks of age were reported to show hypertension and reduced glomerular filtration rate at 6 to 8 weeks in comparison with controls,[13] and Zheng et al[14] performed UNX in mice and found a more advanced diabetic nephropathy in diabetic mice but almost no effects in nondiabetic mice.

Studies on human kidney donors have been limited, because invasive testing is ruled out in the clinical setting and it is

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scarcely more feasible to recruit living donors for the long-term follow-up of key renal functional and morphological parameters. With this background, the objective of the present study was to examine the consequences of UNX in male and female rats, as a model of donor nephrectomy, on BP, renal sodium handling, salt sensitivity, oxidative stress, and renal injury over 18 months and on renal pathology variables gathered at 18 months.

Methods

Animals

Male and female Wistar rats born and raised in the experimental animal service of the University of Granada were used. Experiments were performed according to European Union guidelines for the ethical care of animals. At 6 weeks of age, the rats were divided into 4 groups (n=25 in each), 1 male and 1 female group for UNX and 1 male and 1 female group for sham operation (controls). In the UNX group, total extirpation of the left kidney was performed, leaving the adrenal gland intact. Sham-operated animals were prepared in the same manner, and the left kidney was handled but not removed. Postsurgery, all animals had access to standard chow and tap water ad libitum.

Experimental Protocol

The same experimental protocol was repeated in the same 8 rats from each study group at 6, 12, and 18 months after surgery in the following consecutive procedures: (1) for baseline, animals were housed in metabolic cages (Panlab, Barcelona, Spain) with food and tap water available ad libitum for a 4-day period (2 days for adaptation+2 experimental days), during which food and fluid intakes were measured and urine samples were collected; (2) for water deprivation, animals were water deprived for 24 hours; (3) for isotonic saline load, animals received an intraperitoneal injection of 0.9% NaCl at 3 mL/100 g of body weight; urine samples were taken at 5 hours after the injection, applying light suprapubic pressure before and after each urine collection to ensure that the bladder was emptied; (4) for hypertonic saline load, animals received an intraperitoneal injection of 3% NaCl at 3 mL/100 g of body weight; and (5) for high-salt intake, animals received 1% NaCl saline solution as sole drinking liquid for 2 weeks. Tail systolic BP was measured before and after 2 weeks of 1% NaCl solution administration, using tail-cuff plethysmography in unanesthetized rats (LE 5001-Pressure Meter, Letica SA, Barcelona, Spain). Data were gathered on the urine volume and total urinary sodium and potassium at all these procedures and on creatinine, proteinuria, *N*-acetyl-β-D-glucosaminidase (index of renal injury), and isoprostanes (as index of oxidative stress) under baseline conditions and after 2 weeks of increased saline intake.

At 18 months, after completion of the above sodium-handling study, all rats were anesthetized with ethyl ether, and blood samples were taken by aortic puncture to determine plasma values of urea, creatinine, total proteins (index of volume expansion), electrolytes (sodium and potassium), total cholesterol, high-density lipoprotein, and low-density lipoprotein. Finally, the rats were killed by exsanguination under ethyl ether anesthesia, and the kidneys and ventricles were removed and weighed. Kidney samples were taken for the pathology study and to measure tissue levels of transforming growth factor-β, type III collagen, nitrate/nitrite, and 8-isoprostane. Analytical procedures, histopathologic analysis, and statistical analysis are described in the online-only Data Supplement.

Results

Morphological Variables

Body weight was similar between control and UNX groups at 6 and 12 months but was significantly reduced in both male and female UNX groups at 18 months. Absolute kidney and heart weights were significantly lower in female than in male rats, and the kidney weight/body weight ratio was higher in the UNX groups than in the control groups, as expected. Compensatory renal hypertrophy was greater ($P<0.05$) in male (159.4±5%) than in female (137.7±4%) UNX rats. Heart weight/body weight and left ventricular weight/body weight ratios were higher in the female groups than in the male groups, and the left ventricular weight/body weight ratio was higher in female UNX rats than in female controls (Figure S1 and Table S1 in the online-only Data Supplement). F1,T1

Blood Pressure

Figure S1 (right) in the online-only Data Supplement shows that the tail systolic BP at 6 months after UNX was higher in the male UNX group than in the male control group, whereas no significant difference was found between the female UNX group and female control group. At 12 and 18 months postsurgery, the systolic BP was higher in the male and female UNX groups than in the respective control groups at 12 and 18 months, and it was higher in the male versus the female UNX group and in the male versus the female control group at 18 months.

Renal Sodium Handling

Baseline water and sodium excretion levels did not differ between UNX and control groups of either sex. No difference in acute renal sodium handling was observed between UNX and control groups of either sex at any time point. Diuresis

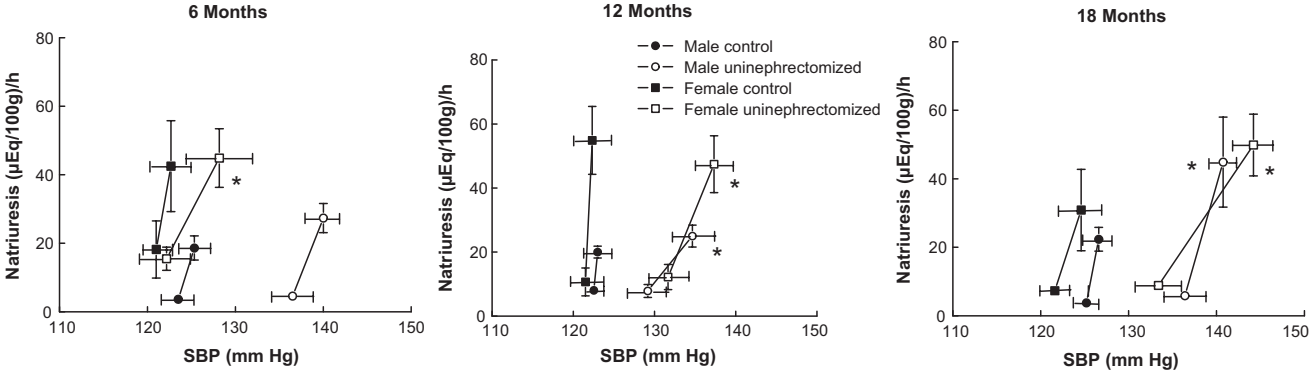

Figure 1. Renal function curves during high- and low-sodium conditions, illustrating the relationship between systolic blood pressure (SBP) and urinary sodium output. All values are expressed as mean±SEM (n=8, each group). The slopes of the relationship between SBP and natriuresis were significantly (*$P<0.05$) blunted in uninephrectomized rats vs male or female controls.

Table. Histopathologic Variables and Morphometrical Analysis of Renal Fibrosis at the End of the Experiment

	Male Control		Female Control		
Morphological Lesions	Control (n=19)	Uninephrectomized (n=17)	Control (n=16)	Uninephrectomized (n=9)	*P Value*
Gomerular sclerosis	0.23±0.43	0.68±0.67	0.00±0.00	0.55±0.52	†,‡
Mesangium increased	0.23±0.43	0.89±0.31	0.12±0.34	0.66±0.50	††,‡
Synechias	0.94±0.24	0.97±0.26	0.87±0.34	1.00±0.00	NS
Cellularity	1.00±0.00	1.00±0.00	0.62±0.50	1.00±0.00	*
Glomerular cyst	0.58±0.50	0.73±0.87	0.18±0.40	0.22±0.44	*
Hyalin casts	0.82±0.52	1.78±0.90	0.12±0.34	0.55±0.52	**,††,§§
Tubular cyst	0.00±0.00	0.92±0.75	0.00±0.00	0.00±0.00	††,§§
Brush border loss	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	NS
Tubular atrofia	0.11±0.33	0.60±0.71	0.00±0.00	0.11±0.33	†
Inflammatory infiltrate	0.17±0.39	0.92±0.67	0.00±0.00	0.11±0.33	†,§
Hyaline arteriolosclerosis	0.41±0.50	1.10±0.45	0.37±0.50	0.66±0.50	†
Renal fibrosis, %	11.92±4.46	19.54±6.07	9.45±2.99	12.53±2.36	††,‡,§§

Values are expressed as mean±SD. NS indicates not significant.
*P<0.05 and **P<0.01 between male and female controls.
†P<0.05 and ††P<0.01 male control and male uninephrectomized.
‡P<0.05 and ‡‡P<0.01 female control and female uninephrectomized.
§P<0.05 and §§P<0.01 male and female uninephrectomized. Test used Mann-Whitney.

and natriuresis were higher in female versus male groups at all time points (Figure S2 in the online-only Data Supplement), although significance was not reached in some tests.

Figure 1 depicts the pressure-natriuresis relationship (between systolic BP and steady-state sodium excretion) in the groups before and after 2 weeks of high-salt intake. The renal function curve was altered in comparison with controls in the male UNX group at 6 months and in both UNX groups at 12 and 18 months, with a shift to the right of the pressure-natriuresis relationship. In addition, a reduced slope was

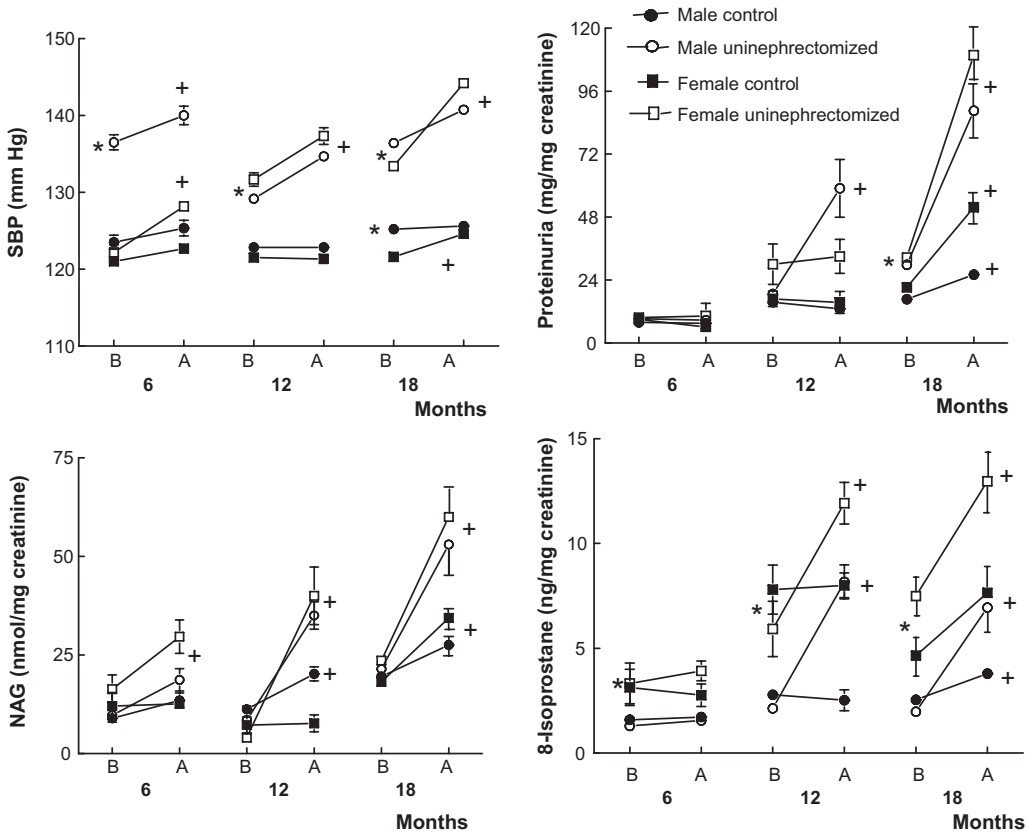

Figure 2. Baseline levels and changes in systolic blood pressure (SBP), proteinuria, *N*-acetyl-β-D-glucosaminidase (NAG), and total excretion of isoprostanes induced by an increased saline intake for 2 weeks (via drinking water, 1% NaCl). Data are mean±SEM (n=8, each group). +P<0.05 vs baseline; *P<0.01 vs male or female controls.

observed in the UNX female group at 6 months and in both UNX groups at 12 and 18 months in comparison with their respective controls.

Plasma and Renal Variables Measured at the End of the Study

Plasma urea and creatinine levels were higher in both UNX groups. Plasma sodium and potassium levels were similar among all groups, whereas total plasma protein was slightly lower in the UNX groups but without reaching statistical significance. Creatinine clearance was reduced in both UNX groups in comparison with their respective controls. After 2 weeks of increased saline intake, creatinine clearance was similar to baseline values in both male groups but markedly elevated versus baseline in the female groups. Total cholesterol was lower in the female UNX groups than in either male group, whereas high-density lipoprotein and low-density lipoprotein were lower in the female control group than in either male group (Table S2 in the online-only Data Supplement).

Salt-Sensitivity Variables

Figure 2 depicts the salt-sensitivity results in the 4 groups. The 2-week high-saline intake period produced a BP increase in male and female UNX groups at different ages (6, 12, and 18 months) but produced no BP increase in male controls and an increase in female controls only at 18 months.

Proteinuria was increased in all groups at 12 and 18 months in comparison with values at 6 months, and the increase was higher in the UNX groups at the end of the experiment. Proteinuria was not significantly modified by the 2-week high-saline intake period in any group at 6 months but was increased versus normal conditions in the male UNX group at 12 months and in all groups at 18 months, when the UNX groups showed the highest values. Urinary *N*-acetyl-β-D-glucosaminidase was increased by high-salt intake in male and female UNX rats at 6 months and markedly increased at 12 and 18 months; it was also increased by the high-salt intake in male control rats at 12 months and in both male and female controls at 18 months. Under normal conditions, urinary isoprostane excretion was higher in female groups than in male groups during the entire study period and was higher in the female UNX group than in the other 3 groups at 18 months. 8-Isoprostane values after the high-salt intake period were markedly higher in both UNX groups at 12 and 18 months and were not changed in male or female control groups except for a slight increase at 18 months.

Renal Tissue Inflammatory and Oxidative Stress Variables

Renal transforming growth factor-β, type III collagen, nitrate/nitrite, and 8-isoprostane values at the end of the experiment (18 months) were similar among all groups (data not shown).

Histopathologic and Quantitative Interstitial Fibrosis Results

Some structural morphological changes were found in the control groups of rats at 18 months, observing mild increased mesangial matrix with glomerular sclerosis and tuft synechiae, cystic appearance of some glomeruli with mild tuft atrophy, and the presence of tubular atrophy (Figure S3). Only tubular casts, mild glomerular cyst, and glomerular hypercellularity were significantly increased in 18-month-old male control rats in comparison with female control rats. Overall, glomerular, tubulointerstitial, and vascular lesions were significantly more severe in male UNX rats than in female UNX rats or male control rats at 18 months of age (Table). Histopathologic examination of renal slices from UNX male rats showed major alterations, including abundant tubular casts, marked dilation of tubules and incipient tubular atrophy, and focal inflammatory infiltrate (Figure S3). In comparison with the other groups, male UNX rats showed more intense glomerular sclerosis, mesangial matrix, tubular atrophy, and the presence of tubular casts, with images of thyroidization and thickening of the basement membrane in both tubular and capsular structures and more severe chronic inflammatory infiltrate and hyaline arteriopathy.

Quantitative interstitial fibrosis analysis revealed a higher degree of fibrosis in both UNX groups versus their respective controls and in male versus female UNX rats, as shown in the Table. The percentage of interstitial connective tissue in the male control rats was higher than the 5% to 8% generally reported in young rats (2–4 months), indicating the development of age-related fibrosis in the kidney of normal rats.

Discussion

The main findings of this study were that UNX after completion of nephrogenesis was accompanied in the long-term by increased BP, proteinuria, and biochemical and pathological signs of renal injury in male and female rats. UNX rats also showed increased salt sensitivity after 2 weeks of an increased saline intake, manifested by augmented BP and an increase in proteinuria and urinary *N*-acetyl-β-D-glucosaminidase (both indexes of renal injury) and isoprostanes (index of oxidative stress). However, UNX did not affect the acute renal sodium handling after saline loads. Moreover, the increased BP appeared earlier and the morphological renal lesions were greater in male than in female UNX rats.

The plasma levels of urea and creatinine were higher and the creatinine clearance was lower in both UNX male and female groups versus controls at 18 months, consistent with their reduced renal mass and in agreement with reports in rats at 6 to 8 weeks[13] and cats at 2 to 5 years[15] after UNX. Creatinine clearance after 2 weeks of increased saline intake was similar to baseline values in both male groups but was markedly elevated in the female groups. This phenomenon has also been reported in rats after a longer period of increased saline intake.[13,16]

Brenner et al[17] proposed an inverse relationship between nephron number and the risk of hypertension in later life. However, controversial data have been reported on the effects of nephrectomy on BP for live kidney donation, with some authors finding no increase in the prevalence of hypertension[1,5,18] and others reporting an increase in BP.[8,19,20] Our results show that UNX produces a long-term BP increase that is sex related, with male rats being more sensitive to renal mass reduction. Our data agree with previous observations of a relationship between reduced nephron number and elevated BP in animal models[21,22] and in humans.[8,19,20] Our data also agree with the

sex differences in BP regulation documented in several animal models.[23] Although the present study did not explore how this sexual dimorphism is produced, one possibility is a different regulation of vascular function between the sexes. Thus, it is known that the responsiveness to vasoconstrictors in the renal vasculature is greater in male groups than in female groups.[24]

Most studies have revealed an increased proteinuria after UNX,[7,11,19] although the amount of urinary protein is usually small. A meta-analysis that included >5000 donors reported a slight increase in proteinuria in comparison with controls,[20] whereas several studies of donors[7,11,19] showed greater proteinuria in the men than in the women. Our study shows that proteinuria was increased in all groups with age and that UNX increased proteinuria in male groups at 12 months, with the greatest increase being observed in both UNX groups at the end of the experiment. Our results agree, in part, with the above data in UNX humans, although we did not find a greater proteinuria in the male groups. It was reported that proteinuria in cats at 2 to 5 years after UNX did not significantly differ from that in age- and sex-matched controls,[15] suggesting species-related differences.

UNX did not affect water and sodium excretion in our male and female rats under baseline conditions or after the different tests at the different ages. However, a reduced natriuretic response to volume expansion was observed in UNX rats at 10 and 26 weeks after surgery,[25] whereas Carlström et al[13] reported an increased diuresis in UNX male rats at 6 to 8 weeks after the operation. These discrepancies may be because of the different post-UNX periods considered.

The kidneys play a key role in the homeostatic regulation of body fluid volume and therefore in long-term BP control.[26] In all forms of chronic hypertension, the renal pressure-natriuresis mechanism is abnormal.[27] Analysis of the relationship between the BP and steady-state sodium excretion in the groups before and after 2 weeks of high-salt intake showed a shift to the right and a reduced slope, resulting in a blunted pressure natriuresis, in the male UNX rats at 6 months and in both UNX groups at 12 and 18 months. These data agree with previous reports[13,14] in UNX male rats after a shorter postoperative period (5–8 weeks). The mechanisms underlying this phenomenon and the salt-sensitive hypertension in the UNX rats have not been addressed in this study, but a role may be played by a decreased dopamine-induced natriuresis[25] or an altered adaptive change of the renal sodium transporters to the increased salt intake of the UNX rats.[28]

We examined the effects of UNX at a young age, but after completion of nephrogenesis, on BP after 2 weeks of increased saline intake, finding that BP was increased in both male and female UNX groups at 6, 12, and 18 months but in female controls only at 18 months. Carlström et al[13] also observed that high-salt diet increased the BP in male groups after a short period of UNX (6–8 weeks). Our study clearly demonstrates that aging in female rats and a reduction in nephron number in male or female rats cause salt-sensitive hypertension. Moreover, variables related to salt-induced renal injury, such as proteinuria, *N*-acetyl-β-D-glucosaminidase, and isoprostanes,[16,29] were markedly increased by the high-salt intake in both UNX groups, indicating that UNX increases renal salt sensitivity.

Several studies have extensively detailed morphological changes in the kidney during aging.[30,31] The normal aging process leads to slightly more pronounced changes in kidney morphology in male versus female rats.[31] Old intact male rats develop glomerulosclerosis, but castrated male rats are protected from this injury.[31] The present results are compatible with previous descriptions of age-related kidney changes and with the sexual dimorphism pattern of these morphological features.[30,31] Our data also reveal more intense histopathologic tubulointerstitial renal lesions and a higher degree of interstitial fibrosis in both UNX groups versus controls and in the male versus female UNX rats.

In summary, this study indicates that UNX in young male and female rats over the long term is associated with increased BP, augmented plasma levels of urea and creatinine, proteinuria, and morphological signs of renal injury. UNX is also associated with salt sensitivity, manifested by an elevated BP, increased oxidative stress, and signs of renal injury in response to an increased saline intake. However, UNX did not affect the acute renal sodium handling. BP was increased earlier and renal parenchyma injury was greater in male versus female UNX rats.

Perspectives

The marked increase in living kidney donation worldwide means that thousands of healthy individuals become uninephric every year. The observations reported in this study contribute experimental data on the long-term consequences of UNX in a rat model of donor nephrectomy. UNX at a young age produced a long-term increase in BP and in biochemical and morphological signs of renal injury in both male and female rats, with an earlier BP elevation and more severe morphological lesions in male versus female UNX rats. This study also showed that UNX causes salt-sensitive hypertension in both sexes. From a clinical and public health perspective, this study indicates that UNX increases BP and aggravates age-related renal injury, especially in male individuals, suggesting that salt restriction should be recommended to attenuate these alterations.

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Disclosures

None.

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Novelty and Significance

What Is New?

- This study analyzes, for the first time, the long-term consequences of uninephrectomy, as a model of donor nephrectomy, on blood pressure, renal function, and morphology and the influence of sex on these variables.

What Is Relevant?

- These findings on the long-term effects of uninephrectomy may be of clinical relevance, given the marked increase in living kidney donations worldwide.

Summary

- This study indicates that uninephrectomy in young male and female rats produces blood pressure elevation, salt sensitivity, and aggravation of age-related renal injury and that the male groups are more susceptible than the female groups to the increase in blood pressure and renal injury.

ONLINE SUPPLEMENTS

Long-term Consequences of Uninephrectomy in Male and Female Rats

Rodríguez-Gómez et al. Effects of Uninephrectomy in rats

Isabel Rodríguez-Gómez, Rosemary Wangensteen[3], Rocío Pérez-Abud[2], Andrés Quesada[3], Raimundo G. del Moral[4], Antonio Osuna[2], Francisco O'Valle[4], Juan de Dios Luna[1] and Félix Vargas[1]

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Supplementary Methods

Analytical procedures. Plasma and urinary electrolytes and creatinine were measured in an autoanalyzer (Hitachi-912, Roche, Spain). Collagen type III was determined with an ELISA kit purchased from Cusabio Biotech. TGF-beta was measured by Luminex x-MAP technology with a kit purchased from Millipore (Billerica, MA, USA), and 8-isoprostane concentrations in urine and kidney samples were measured by a Colorimetric Assay Kit purchased from Cayman Chemical (Cayman Ann Arbor, MI, USA). Proteinuria was determined with the DC Protein Assay kit (Bio-Rad, Madrid, Spain), and urine NAG levels were determined by means of a colorimetric method purchased from Roche Diagnostics (Barcelona, Spain). Tissue NO^-_2 and NO^-_3 (NOx) concentrations were measured by using nitrate reductase and Griess reaction. Renal tissue was homogenized in 1 ml of a solution of 0.1M phosphate buffered saline (pH 7.4), 1mM EDTA, and 0.005% butylated hydroxytoluene (BHT).

Histopathological analysis. For conventional morphology, paraffin-embedded longitudinal rat kidney sections in sagittal plane were stained with hematoxylin and eosin, Masson's trichrome, and periodic acid-Schiff stain (PAS). The morphological study was done in blinded fashion (RGM, FO) on 4-micrometer sections with light microscopy, using the most appropriate stain for each lesion. The severity of glomerular (sclerosis, cysts, synechiae, mesangium proliferation, cellularity), tubular (atrophy, cysts, brush border loss, casts, inflammatory infiltrate), and vascular (hyaline arteriolosclerosis, fibrinoid necrosis) lesions was calculated semiquantitatively using a 4-point scale (0, absence; 1, mild [<10% of tubules, vessels or glomeruli involved]; 2, moderate [10-25%]; 3, severe [>25%]). A quantitative study of interstitial fibrosis was performed by image analysis using Sirius red stain and the Fibrosis HR® program, as previously described by our group (1)

Statistical Analysis. Stata 11.1 was used for the statistical analyses. Differences were considered statistically significant at $p<0.05$. The following statistical analyses were performed. 1) The same four-factor repeated-measures design was used for each of the experiments (baseline, WD, ISL, HSL and high salt diet), with sex (female/male), treatment (control/nephrectomized), experiment (baseline, WD, ISL, HSL and high salt diet) and time (6, 12 and 18 months) as fixed effect factors and rat as random effect factor. Sex and treatment factors were crossed, experiment and month were repeated-measures factors, and rats were nested in the sex-treatment interaction. When interactions were significant, pairwise comparisons were performed using Bonferroni´s penalization. When appropriate, logarithm transformations were used. 2) The above design, but restricted to three fixed-effect factors (sex, treatment and time), was used to analyze the time course of body weight and blood pressure measurements. 3) A four-factor repeated-measures design was used to analyze variables related to salt sensitivity (BP, proteinuria, NAG and isoprostanes), with sex, treatment, time, and condition (before/after salt) as fixed-effect factors and rat as random-effect factor. Sex and treatment factors were crossed, condition and month were repeated-measures factors, and rats were nested in the sex-treatment interaction. When interactions were significant, pairwise comparisons were performed using Bonferroni´s penalization. 4) Slopes of the relationships between RPP and diuresis and natriuresis were computed for each rat, and the mean of slopes was obtained for each group. One-way ANOVA was performed to compare the means of slopes, followed by pairwise comparisons using Tukey´s method. 5) A two crossed-factor design (sex and treatment) was used to study

measures at the study endpoint. Sex-treatment interactions were analyzed and, when significance was obtained, pairwise comparisons were made using Bonferroni's penalization because of the unbalanced sample sizes. 6) The non-parametric Kruskal-Wallis test and Mann Whitney U-test were used to analyze morphometric variables.

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Supplementary Table

Table S1. Morphologic variables in the experimental groups at the end of the study.

	Male		Female	
Variables	**Control** (n=19)	**Uninephrectomized** (n=17)	**Control** (n=16)	**Uninephrectomized** (n=9)
FBW (g)	657.35±20.3	577.63±17.0*	340.31±16.9††	307.56±16.9*
KW (mg)	1425.8±65.6	1981.4±77.1**	834.8±30.7††	1072.0±61.3**
HW (mg)	1325.0±36.5	1258.7±31.6	875.5±35.9††	869.9±53.1
LVW (mg)	1063.8±29.1	1019.5±27.2	708.2±31.4††	722.4±47.7
KW/BW (mg/g)	2.19±0.11	3.47±0.16**	2.54±0.13†	3.50±0.11*
HW/BW (mg/g)	2.03±0.05	2.20±0.07	2.62±0.10†	2.84±0.10
LVW/BW (mg/g)	1.63±0.04	1.78±0.05	2.12±0.08†	2.35±0.06*
LVW/HW	0.80±0.007	0.81±0.010	0.81±0.010	0.83±0.024

Data expressed as means ± s.e.m. FBW, final body weight; KW, final kidney weight; HW, final heart weight; LVW, final left ventricular weight; KW/BW, kidney weight versus body weight ratio; HW/BW, heart weight versus body weight ratio; LVW/BW, left ventricular weight versus body weight ratio; LVW/HW, left ventricular weight versus heart weight ratio. * $P<0.05$, ** $P<0.01$, vs their respective control group. † $P<0.05$, ††$P<0.01$, vs the control male group.

Table S2. Plasma and renal variables measured at the end of the study in the experimental groups.

	Male		Female	
Variables	**Control** (n=19)	**Uninephrectomized** (n=17)	**Control** (n=16)	**Uninephrectomized** (n=9)
Na (mEq/L)	148.44±0.85	146.00±1.46	146.75±1.08	145.33±1.05
K (mEq/L)	4.08±0.08	4.17±0.19	3.87±0.14	4.10±0.10
Urea (mg/dL)	31.59±1.42	40.81±3.76*	34.25±2.21	42.29±2.58*
Creatinine (mg/dL)	0.31±0.01	0.44±0.05*	0.27±0.01	0.44±0.08*
Total Proteins (g/dL)	6.41±0.08	6.21±0.17	6.52±0.11	6.28±0.02
T. Cholesterol (mg/dL)	139.21±6.56	146.77±16.5	111.56±8.41	101.89±0.95†
HDL (mg/dL)	81.80±3.28	79.73±3.87	65.78±4.29†	74.31±2.49
LDL (mg/dL)	7.98±0.90	9.53±1.56	3.95±0.65†	4.98±1.33
CrC (ml/min.100g) c.n	0.13±0.01	0.07±0.02*	0.29±0.02†	0.12±0.01*
CrC (ml/min.100g) post salt	0.12±0.01	0.05±0.02	0.44±0.20‡	0.56±0.16‡

Data expressed as means ± s.e.m. CrC c.n, creatinine clearance in normal conditions; CrC post salt, creatinine clearance post treatment of salt. * P<0.01 vs the control group. † P<0.01 vs the male control group. ‡ vs normal conditions.

Supplementary Figures and Legends.

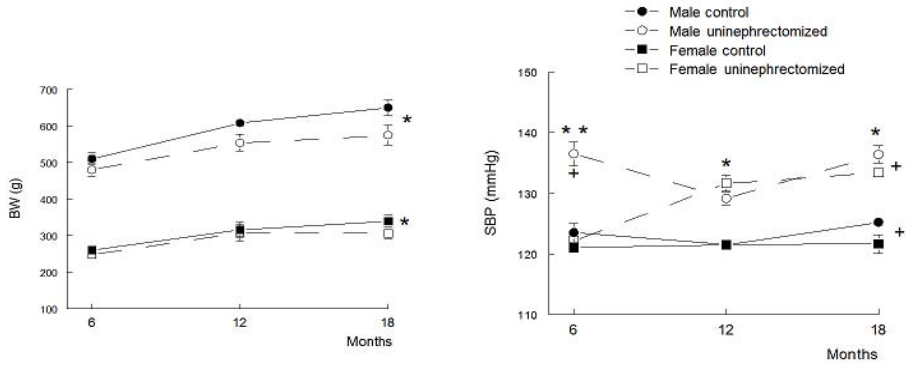

Figure S1: (Left panel) Time course of body weight (BW) and tail systolic blood pressure (SBP) in the groups measured by plethysmography (Right panel). (n=8, each group) Data are means ± SEM (n=8, each group). * p<0.05; ** p<0.01 *versus* male and female controls; + p<0.05 *versus* female or female uninephrectomized groups

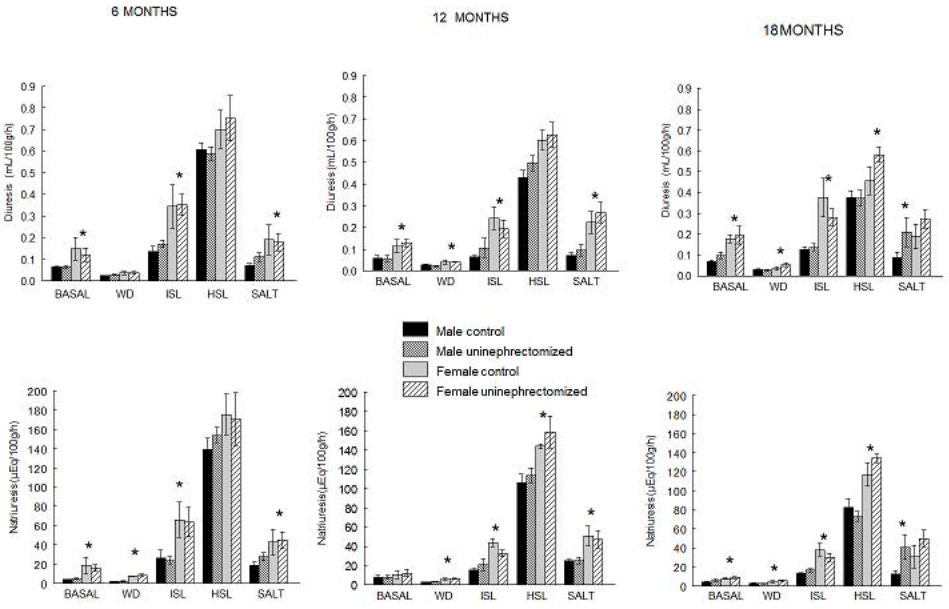

Figure S2.- Diuresis and natriuresis in the experimental groups under baseline conditions and after several stresses. WD, water deprivation for 24 h, ISL, isotonic saline load (1% NaCl, 3 ml/100g, ip), HSL, hypertonic saline load (3% NaCl, 3 ml/100g, ip), after two weeks of increased saline intake (SALT, 1% NaCl via drinking water). All values are expressed as mean±SEM (n=8 each group) *$p<0.05$ *versus* male controls or male uninephrectomized rats.

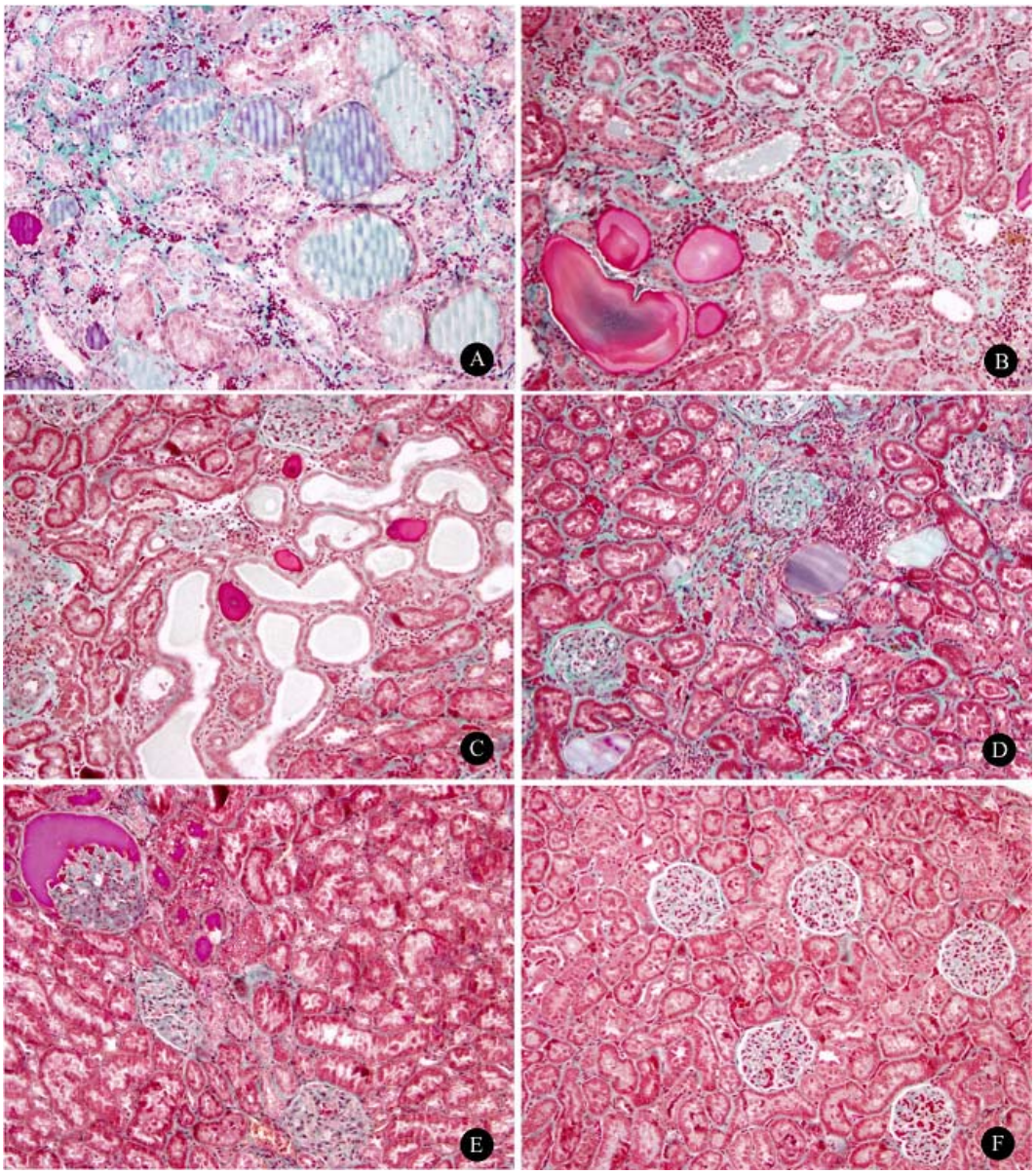

Figure S3.- Kidney lesions in uninephrectomized 18-month-old rats. A and C) Multiple tubular casts (arrowhead), tubular dilation (asterisk) and mild inflammatory infiltrate in male uninephrectomized rat. B and D) Glomerular sclerosis (arrow) and fewer tubular casts in female uninephrectomized rat. E) Some tubular and glomerular casts in control male rat. F) Normal feature of renal cortex in control female rat (Masson's trichrome, original magnification x10).

How Safe Is Unilateral Nephrectomy?

Joseph I. Shapiro and Larry D. Dial

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Editorial Commentary

How Safe Is Unilateral Nephrectomy?

Joseph I. Shapiro, Larry D. Dial

See related article, pp 1458–1463

In this issue of *Hypertension*, Rodríguez-Gómez et al[1] report on their studies of the long-term consequences of uninephrectomy (UNx) in male and female rats. This is an excellent study with very interesting implications to science, as well as clinical practice. The investigators studied the effects of UNx over 18 months, a long period of time given the relatively short life span of the rat. They noted that the males appeared to be more sensitive to the UNx as evidenced by earlier salt-sensitive hypertension, as well as greater pathological changes when animals were studied at the 18-month time point. We point out that the methods used by the authors to determine salt sensitivity were quite clever. Both acute and chronic salt loading were used to assess the relationships between blood pressure and natriuresis, the Guytonian renal function curves. Interestingly but not surprisingly, the authors observed that the presence of salt-sensitive hypertension corresponded quite well with renal pathology in the rats studied at 18 months.[1]

The major finding reported by Rodríguez-Gómez et al[1] was that the males seemed to adapt less well to UNx than the female rats. This observation is consistent with a body of work addressing sex differences. Using micropuncture and other physiological measurements, Baylis and Wilson[2] demonstrated that glomerular capillary hydrostatic pressure was substantially higher in male rats compared with female rats subjected to UNx and a high-protein diet. Mulroney et al[3] demonstrated considerably less hypertrophy after UNx in female when compared with male rats. This group also observed that testosterone appeared to be the driving force in this differential hypertrophy. Unfortunately, the current study was not able to address the role that hormones play in the observed differences in salt sensitivity.

The findings of this article force one to reflect on the relationship between salt and hypertension in clinical subjects. First and foremost, there has been some debate as to whether the kidney is truly the controller of blood pressure as proposed by Crawford et al[4] some years ago. Although a detailed review of this debate is well beyond the scope of this commentary, most have concluded that the concepts developed by Guyton are applicable to a considerable portion of both experimental and human hypertension.[5] However, the relationship between salt and blood pressure is certainly more complex than predicted by these concepts. This has been demonstrated, perhaps best, in the famous Intersalt study. In this landmark article, a rather weak correlation was seen between dietary salt intake and systolic blood pressure, which was, in fact, lost when the centers representative of the very lowest salt intakes were dropped from the analysis. However, the increases in systolic blood pressure that occurred with age were closely related to dietary salt intake.[6] In short, increasing dietary salt intake does appear to result in salt sensitivity over long periods of time. The tacit assumption is that, clinically, salt sensitivity corresponds with pathological changes in the kidney. The molecular mechanisms underlying this are topics of intense investigation. Whether and how sex differences might weigh in on the relationship between dietary salt and progressive increases in salt sensitivity are still opaque.

One obvious and important clinical implication of UNx is kidney transplant donation. For many years, we have understood that, provided extensive screening is performed on the donor, kidney donation can be considered safe. That said, it has been clearly established that, even with appropriate screening, kidney donation appears to lead to increases in the incidence of hypertension and proteinuria.[7] If the data from Rodríguez-Gómez et al[1] is applicable in humans, this would suggest that female sex might confer additional safety to the prospective donors. Fortunately, the risk of renal donors developing end-stage renal disease with currently accepted screening criteria is extremely small, although some degrees of proteinuria and increased risk of hypertension have been noted in several series.[8] Although it is difficult to exclude any difference between female and male donors, these differences are less than one might anticipate if we extrapolate the findings of the article under discussion. One reason for this may be age. Typically, human donors are mature, whereas the animals studied by Rodríguez-Gómez et al[1] were quite young. It has been clearly established that renal hypertrophy after UNx decreases in both experimental animals and humans with age, and this hypertrophy does appear closely linked to the processes that lead to salt-sensitive hypertension, proteinuria, and progressive renal failure.[9]

In summary, Rodríguez-Gómez et al[1] provide us with an interesting study of how sex differences produce considerable variance in the physiological response to UNx. Further work will be necessary to fully understand the hormonal and molecular mechanisms operant in these differences, as well as the clinical implications of their findings.

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Disclosures

None.

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PLoS one

Urinary Aminopeptidase Activities as Early and Predictive Biomarkers of Renal Dysfunction in Cisplatin-Treated Rats

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Abstract

This study analyzes the fluorimetric determination of alanyl- (Ala), glutamyl- (Glu), leucyl-cystinyl- (Cys) and aspartyl-aminopeptidase (AspAp) urinary enzymatic activities as early and predictive biomarkers of renal dysfunction in cisplatin-treated rats. Male Wistar rats (n = 8 each group) received a single subcutaneous injection of either saline or cisplatin 3.5 or 7 mg/kg, and urine samples were taken at 0, 1, 2, 3 and 14 days after treatment. In urine samples we determined Ala, Glu, Cys and AspAp activities, proteinuria, *N*-acetyl-β-D-glucosaminidase (NAG), albumin, and neutrophil gelatinase-associated lipocalin (NGAL). Plasma creatinine, creatinine clearance and renal morphological variables were measured at the end of the experiment. CysAp, NAG and albumin were increased 48 hours after treatment in the cisplatin 3.5 mg/kg treated group. At 24 hours, all urinary aminopeptidase activities and albuminuria were significantly increased in the cisplatin 7 mg/kg treated group. Aminopeptidase urinary activities correlated ($p<0.011$; $r^2>0.259$) with plasma creatinine, creatinine clearance and/or kidney weight/body weight ratio at the end of the experiment and they could be considered as predictive biomarkers of renal injury severity. ROC-AUC analysis was made to study their sensitivity and specificity to distinguish between treated and untreated rats at day 1. All aminopeptidase activities showed an AUC>0.633. We conclude that Ala, Cys, Glu and AspAp enzymatic activities are early and predictive urinary biomarkers of the renal dysfunction induced by cisplatin. These determinations can be very useful in the prognostic and diagnostic of renal dysfunction in preclinical research and clinical practice.

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Introduction

Acute kidney injury (AKI) is a common clinical problem that is defined by an abrupt increase in serum creatinine over 48 h resulting from injury or insult that causes a functional or structural change in the kidney. The main cause of AKI is the acute apoptosis or necrosis of renal tubular cells, and the RIFLE (risk, injury, failure, loss, and end-stage renal disease) classification scheme is based in criteria of serum creatinine to classify renal dysfunction in patients [1].

Nevertheless, traditionally used markers of AKI, such as blood urea nitrogen (BUN) and creatinine are insensitive, nonspecific, and do not adequately differentiate between the different stages of AKI [2]. Serum creatinine accumulates over time, and changes in creatinine concentrations become apparent only when the kidneys have lost 50% of their functional capacity [3,4].

Early detection of AKI remains a challenge in both preclinical research and clinical practice. There is an urgent need for better biomarkers to permit more timely diagnosis of AKI, prediction of injury severity, and safety assessment during drug development [2].

Enzymes released from damaged tubular cells and excreted into urine are the most promising biomarkers for an early detection of AKI. They have an obvious diagnostic benefit because their measurements may provide detailed information about the nature, size and site of the damage to tubular cells and their possible necrosis or dysfunction [5]. One of these enzymes, AlaAp (EC 3.4.11.2), is an enzyme of the brush border proposed in the early seventies as a urinary marker of renal disease [6], and there is an automatic photometric assay for its determination [7]. AlaAp, together with GluAp (EC 3.4.11.7), CysAp (3.4.11.3) and AspAp (EC 3.4.11.21) are present in the renal tubular cells [8–10] and they have an aminopeptidasic function in angiotensin II metabolism, peptide that is increased in renal diseases. In our laboratory, we have recently determined the activity of these four aminopep-

tidases as an index of renal damage in salt-treated and hyperthyroid rats [11].

In this work we investigated the reliability of the fluorimetric determination of AlaAp, GluAp, CysAp and AspAp urinary enzymatic activities as biomarkers of renal dysfunction induced by cisplatin at two different doses (3.5 and 7 mg/kg) in rats. Cisplatin is an antineoplastic drug known for its direct proximal tubular nephrotoxicity in both humans and animals [12,13]. Rats treated with one single dose of cisplatin at 7 mg/kg exhibit tubular degeneration and necrosis [14] accompanied with a maximal increase in the excretion of NAG [15], a renal tubular enzyme [16], and glucosuria [15] at the third day of injection. At day 7, plasma creatinine and BUN reach a maximum [15] and tubular epithelial regeneration and dilatation are observed on day 8 after treatment [14]. From day 14 to 56, development of tubulointerstitial fibrosis is observed in these rats with no differences in urinary enzymes or glucose, but a significant increase in plasma creatinine and BUN at day 56 [15].

We studied aminopeptidase activities as early biomarkers of AKI and their ability to detect a slight renal damage evaluating their increased urinary excretion at the first three days from injection. We also analyzed their value as predictive biomarkers of the severity of renal dysfunction by correlating the first day urinary excretion level of the marker with two parameters of renal function (plasma creatinine and creatinine clearance) and two parameters of structural damage (renal hypertrophy and interstitial fibrosis). We determined other parameters commonly used as urinary biomarkers of renal damage (proteinuria, NAG, albumin, and NGAL) and made combined ROC area under the curve (AUC) analysis in order to establish their sensitivity and specificity to detect renal alterations.

Materials and Methods

Ethics statement

All experimental procedures were performed according to the European Union Guidelines to the Care and Use of Laboratory Animals and approved by the Ethical Committee of the University of Jaén with the approval ID R1/12/2010/66.

Animals and drugs

24 male Wistar rats weighing 227–279 g were purchased from Harlan Laboratories (Barcelona, Spain). These rats were kept in a room maintained at 24±1°C and humidity of 55±10%, with a 12-

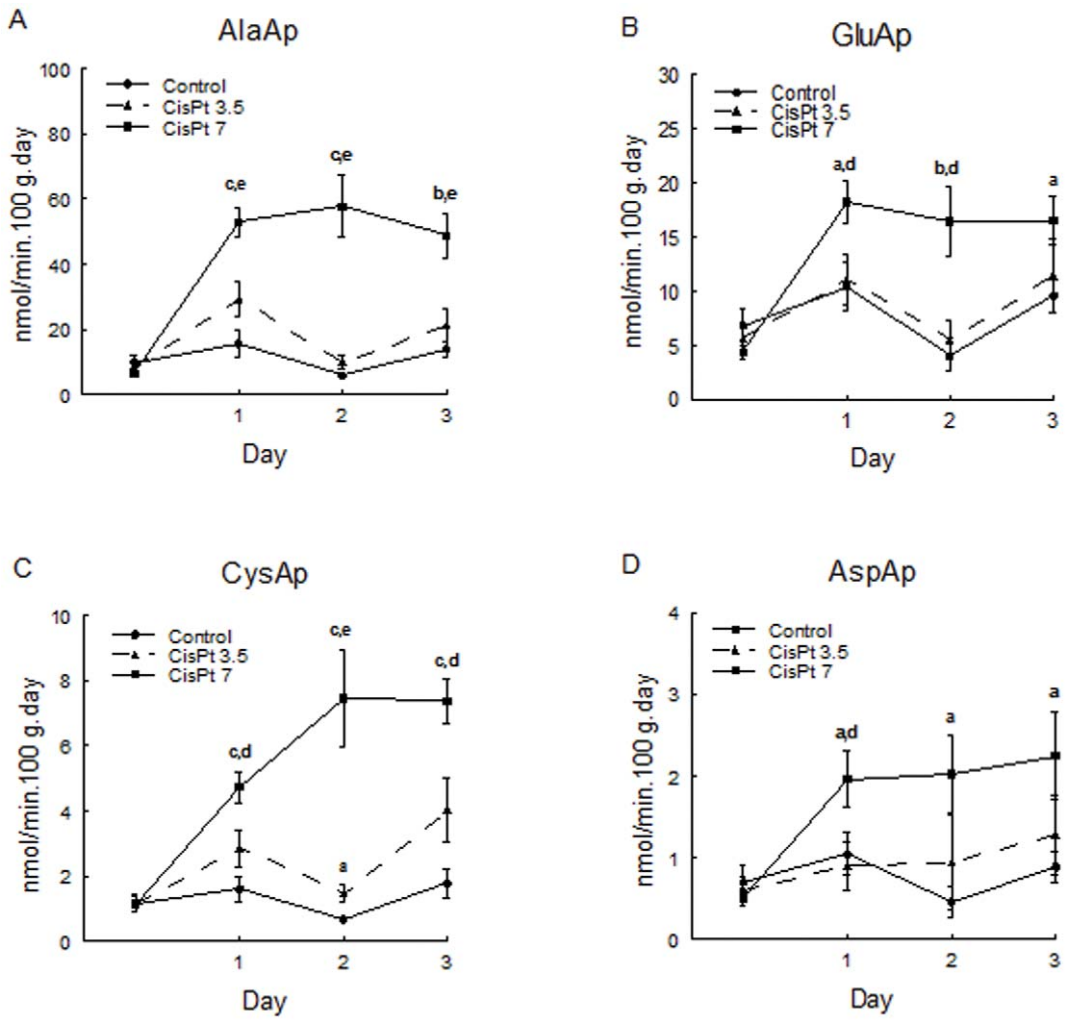

Figure 1. Urinary aminopeptidase activities. AlaAp (A), GluAp (B), CysAp (C), and AspAp (D) urinary activities excreted per day and 100 g of rat at 0, 1, 2 and 3 days of treatment in control, CisPt3.5 and CisPt7 groups. Data are means ± SEM. **a** $p<0.05$, **b** $p<0.01$, **c** $p<0.001$ compared with control group. **d** $p<0.05$, **e** $p<0.01$ compared with CisPt3.5 group.
doi:10.1371/journal.pone.0040402.g001

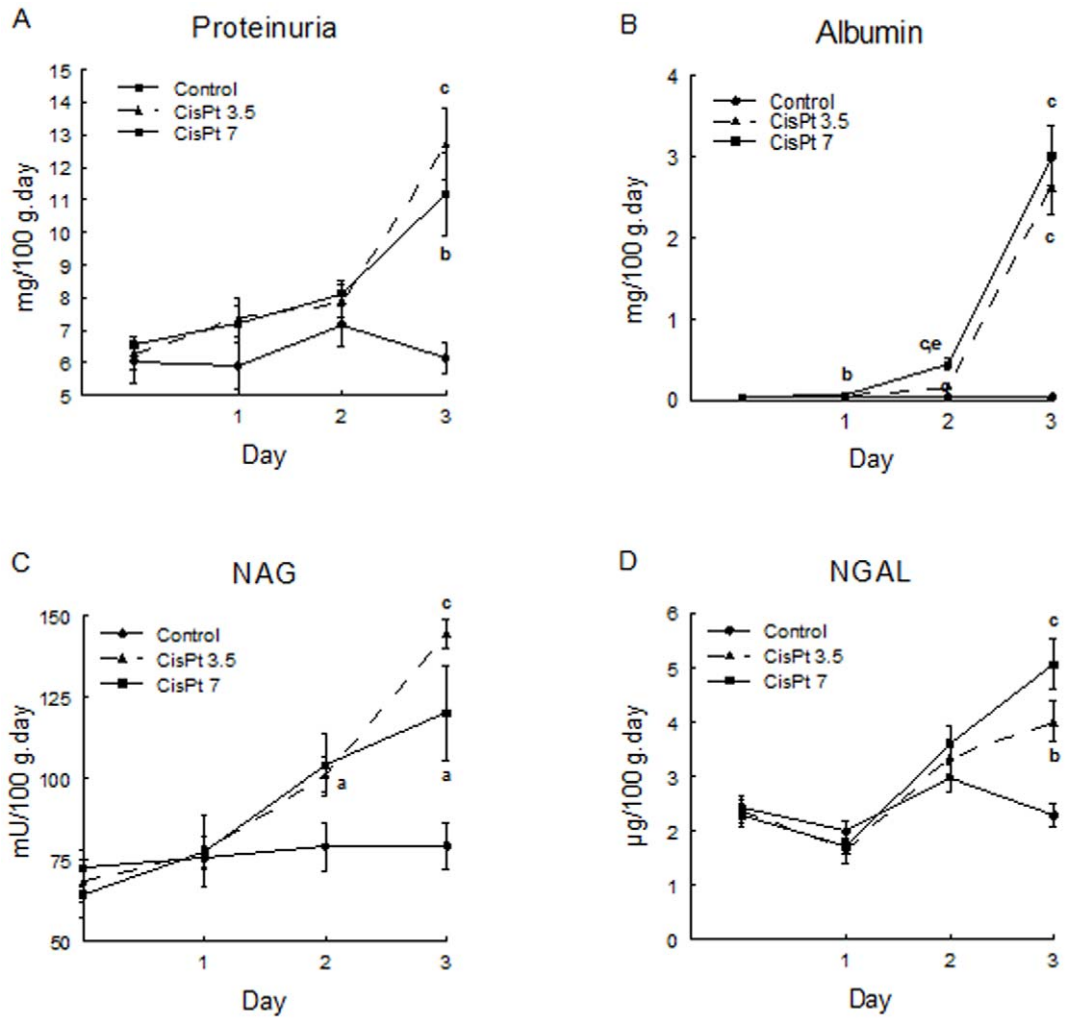

Figure 2. Proteinuria, albuminuria, NAG and NGAL excretion. Proteinuria (A), albuminuria (B), *N*-acetyl-β-D-glucosaminidase (C) and neutrophil gelatinase-associated lipocalin (NGAL) excreted per day and 100 g of rat at 0, 1, 2 and 3 days of treatment in control, CisPt3.5 and CisPt7 groups. Data are means ± SEM. **a** $p<0.05$, **b** $p<0.01$, **c** $p<0.001$ compared with control group. **d** $p<0.05$, **e** $p<0.01$ compared with CisPt3.5 group.
doi:10.1371/journal.pone.0040402.g002

h light/dark cycle and had free access to rat chow and tap water. Cisplatin (Sigma, Madrid, Spain) was dissolved in saline (1.5 and 3 mg/ml).

Experimental protocols

In order to examine the time course enzymatic activities, rats were distributed in three groups: Control, CisPt3.5 and CisPt7, (n = 8 each group), that received a subcutaneous injection of either

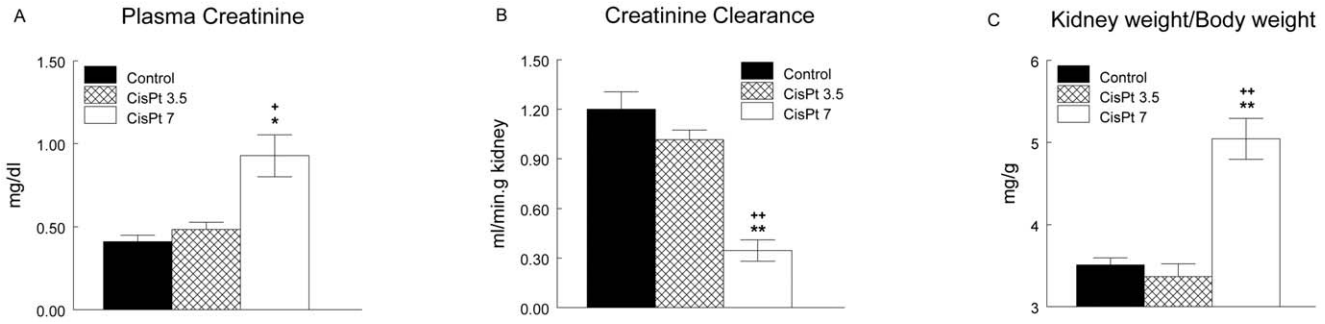

Figure 3. Plasma creatinine, creatinine clearance and kidney weight/body weight ratio at the end of the experiment. Plasma creatinine (A), creatinine clearance (B) and kidney weight/body weight ratio (C) at the end of the experiment in control, Cispt3.5 and CisPt7 groups. *$p<0.01$, **$p<0.001$ compared with control group. + $p<0.01$, ++ $p<0.001$ compared with CisPt3.5 group.
doi:10.1371/journal.pone.0040402.g003

saline or cisplatin 3.5 mg/kg and 7 mg/kg, respectively. One day before treatment and at 0, 1, 2 and 13 days after cisplatin or saline administration, rats were housed in metabolic cages and 24-h urine collection was made, obtaining urine samples at 0, 1, 2, 3 and 14 days. Urine samples were centrifuged 15 min at 1000 g, aliquoted and frozen at −80°C until assay. At the end of the experiment, blood samples were obtained from left ventricle under anesthesia (pentobarbital, 50 mg/kg, i.p.), centrifuged 15 min at 1000 *g*, aliquoted and stored at −80°C. Kidneys were removed and weighted. One kidney was fixed in 10% neutral-buffered formalin solution during 24 h and subsequently placed in 70% ethanol.

Analytical procedures

Ala, Glu, Cys and AspAp urinary activities were determined in duplicate in a kinetic fluorimetric assay using alanyl-, glutamyl-, cystinyl- and aspartyl-β-naphtylamide as substrates, respectively. 20 µl of urine were incubated during 30 min at 37°C with 90 µl of their corresponding substrate solution (2.14 mg/dl alanyl-β-naphtylamide, 10 mg/dl bovine serum albumin (BSA), 10 mg/dl dithiotreitol (DTT) in pH 7.4 50 mM HCl-Tris; 2.72 mg/dl glutamyl-β-naphtylamide, 10 mg/dl BSA, 10 mg/dl DTT and 555 mg/dl $CaCl_2$ in pH 7.4 50 mM HCl-Tris; 5.63 mg/dl cystinyl-β-naphtylamide, 10 mg/dl BSA, 10 mg/dl DTT in pH 6 50 mM HCl-Tris; 2.58 mg/dl aspartyl-β-naphtylamide, 10 mg/dl BSA, 555 mg/dl $CaCl_2$ in pH 7.4 50 mM HCl-Tris). The substrates were previously dissolved in 1 ml of dimethyl sulfoxide and stored at −20°C. The amount of β-naphtylamine released as a result of the aminopeptidase activities was measured fluorimetrically at an emission wavelength of 412 nm with an excitation wavelength of 345 nm, and quantified using a standard curve of β-naphtylamine (0–200 nmol/ml). Sample blanks were made in duplicate using an incubation solution that did not contain the substrate of the enzyme. Fluorimetric data from samples, blanks and standard curve were taken each minute, and fluorescence of sample blanks was subtracted from the fluorescence of the samples at each point. Specific aminopeptidase activities were calculated from the slope of the linear portion of

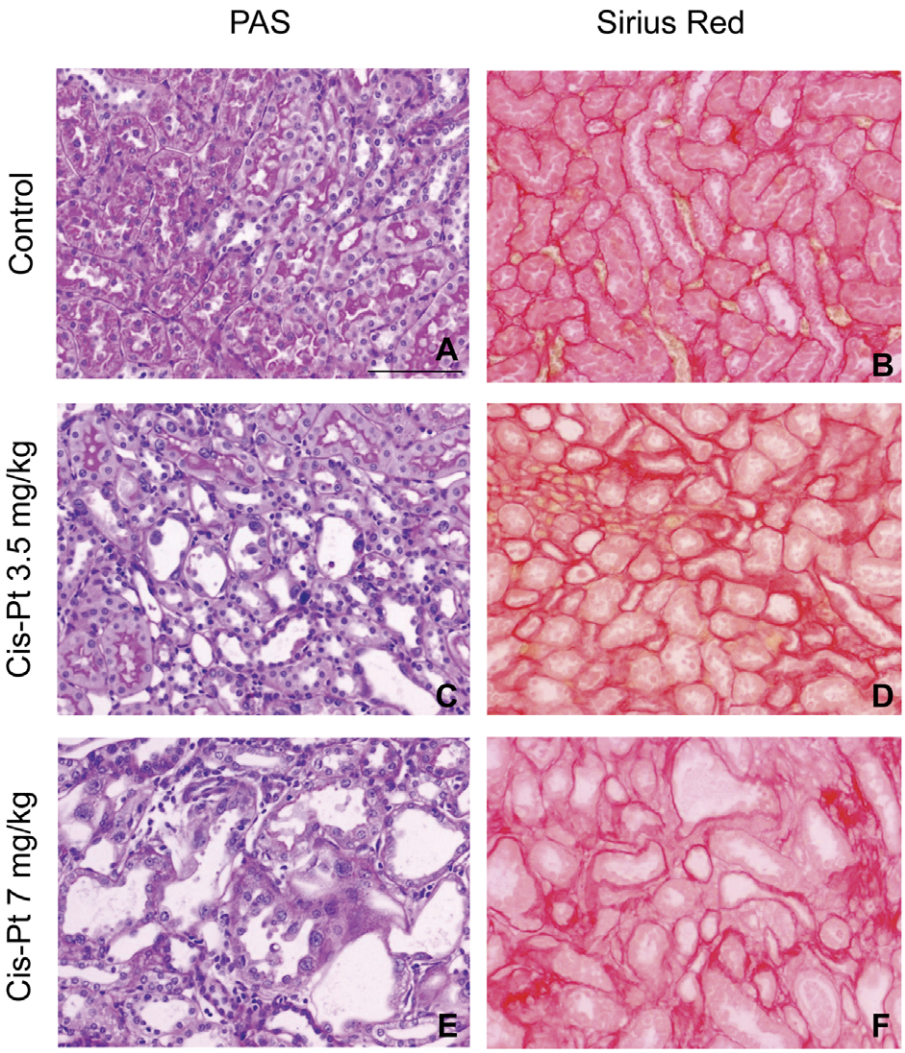

Figure 4. Morphological changes and tubulointerstitial fibrosis in renal outer medulla induced by cisplatin. Left panel shows control group without morphological lesion (A), CisPt3.5 (C) and CisPt7 (E) groups whit moderate nuclear dysplasia, incipient acute tubular necrosis, marked tubular dilation and apoptotic cells in tubular lumen of proximal convoluted tubules in the corticomedullary junction (PAS original magnification x200). Scale bar = 100 micrometers. Right panel shows representative photographs of Sirius red staining of kidneys from control (B), 3.5 (D) and 7 mg/kg cisplatin-treated rats (F) after 14 days (original magnification x200).
doi:10.1371/journal.pone.0040402.g004

Table 1. Morphological and morphometrical variables analyzed in kidney biopsies from Control, CisPt3.5 and CisPt7 rats at the end of the experiment.

Groups	Control	CisPt 3.5	CisPt 7
Displasia S3 segment	0.00 ± 0.00	1.63 ± 0.18**	2.75 ± 0.16**,++
Acute tubular necrosis	0.00 ± 0.00	1.13 ± 0.23*	2.13 ± 0.13**,+
Apoptosis	0.00 ± 0.00	1.25 ± 0.25*	2 ± 0.27**
Tubular casts	0.00 ± 0.0 0	0.13 ± 0.13	1.38 ± 0.26*,+
Tubular atrophy	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.13
Tubular dilation	0.00 ± 0.00	1.00 ± 0.19*	2.75 ± 0.16**,++
Tubular vacuolization	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tubular mitosis	0.00 ± 0.00	0.50 ± 0.19	1.25 ± 0.16**,+
Inflammatory infiltrate	0.00 ± 0.00	0.20 ± 0.20	0.50 ± 0.33
Glomerular lesion	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Vascular lesion	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.20
Fibrosis (%)	2.61 ± 0.28	5.28 ± 0.43**	5.36 ± 0.50**
Fibrosis area (μm^2)	3591 ± 388	7194 ± 548**	7361 ± 660**

*p<0.01, **p<0.001 *versus* control group. +p<0.01, ++p<0.001 *versus* Cispt3.5 group. n = 8 each group.
doi:10.1371/journal.pone.0040402.t001

enzymatic assay obtaining nanomol of substrate hydrolyzed per ml and minute. These values were normalized by diuresis and expressed as absolute excretion per 100 g of rat and day.

Proteinuria was determined with the red pirogallol protein assay (Spinreact, Barcelona, Spain). NAG was determined by a colorimetric method purchased from Roche Diagnostics (Barcelona, Spain). Urinary albumin and NGAL were analyzed by ELISA with kits purchased from Bethyl Laboratories (Montgomery, TX, USA) and Bioporto Diagnostics (Gentofte, Denmark), respectively. Plasma and urinary creatinine was measured by the kinetic method of Jaffé, based on the reaction of creatinine with sodium picrate.

These urinary parameters were measured at 0, 1, 2 and 3 days from injection of cisplatin and expressed as absolute excretion per 100 g of rat and day. Plasma and urinary creatinine concentration were measured at the end of the experiment.

Histopathological analysis

For conventional morphology, buffered 10% formaldehyde-fixed, paraffin-embedded transversal kidney sections in horizontal plane were stained with hematoxylin and eosin, Masson's trichrome and periodic acid-Schiff stain (PAS). The morphological study was done in blinded fashion on 4-micrometer sections with light microscopy, using the most appropriate stain for each lesion. The severity of lesions was calculated semiquantitatively using a 0 to 3 scale (0, absence; 1, mild [<10% of juxtamedullary proximal tubules, vessels or glomeruli involved]; 2, moderate [10 to 25%]; 3, severe [>25%]).

Morphometrical analysis

Kidney samples fixed in buffered 10% formalin were embedded in paraffin and serially sectioned at 5 μm thickness. Afterwards, they were stained with 1% picro Sirius red F3BA (Gurr, BDH Chemicals Ltd., Poole, United Kingdom) for image analysis quantification. To improve staining, tissue sections were kept after deparaffination for 3–5 days in 70% ethanol as mordent. Picro Sirius red stains connective fibers deep red and cell nuclei and cytoplasmatic structures light red/bright yellow [17].

To automatically quantify interstitial connective tissue and glomerular morphometry on rats kidney histologic sections, we developed several image processing algorithms that had been brought together in one image analysis application, named Fibrosis HR® [18]. We evaluated 20 images of corticomedullary junction per kidney as previously described [18].

Statistical analyses

We used *t* test for the analysis of variables with normal distribution. Mann-Whitney W (Wilcoxon) test was used to analyze the differences in morphological variables between treated and control rats. Differences were considered statistically significant at $P<0.05$ level. Linear regression and analysis of variance were made to establish the correlation of urinary biomarkers at the first day with plasma creatinine, creatinine clearance, renal hypertrophy and interstitial fibrosis at the end of the experiment. $P<0.05$ and $|r| > 0.5$ was considered as a strong correlation. ROC-AUC analysis was made with JLABROC4 software [19]. We used the results obtained at 24 hours of treatment, and studied the sensitivity and specificity of each marker to differentiate if a rat had received cisplatin (either 3.5 or 7 mg/kg) or saline.

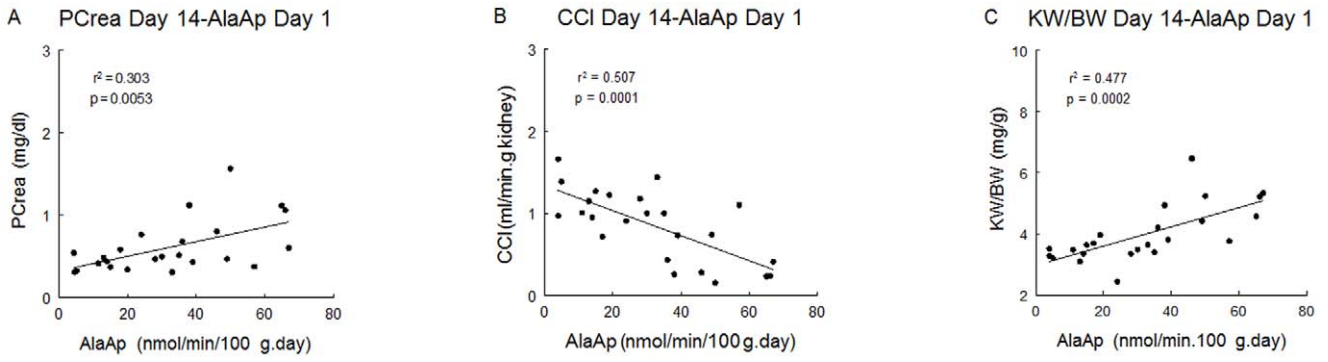

Figure 5. Linear regressions between urinary AlaAp activity at day 1 and plasma creatinine, creatinine clearance and kidney weight/body weight at day 14. Linear regressions between AlaAp activity at day 1 and plasma creatinine (A), creatinine clearance (B) and kidney weight/body weight (C) at the end of the experiment.
doi:10.1371/journal.pone.0040402.g005

Table 2. P-value and correlation coefficients (r and r^2) of linear regression between urinary biomarkers excreted at day 1 *versus* kidney weight/body weight ratio, interstitial fibrosis, plasma creatinine and creatinine clearance at day 14.

	P value	R	R^2
Kidney weight/body weight			
AlaAp*	0.0002	0.690	0.477
CysAp*	0.0009	0.632	0.400
GluAp*	0.0040	0.565	0.319
AspAp*	0.0110	0.509	0.259
NGAL	0.2877	−0.226	0.051
Proteinuria	0.4219	0.172	0.030
NAG	0.7564	0.067	0.447
Albumin	0.7056	0.081	0.007
Interstitial fibrosis			
AlaAp	0.0621	0.386	0.149
Albumin	0.1473	0.305	0.093
CysAp	0.2410	0.249	0.062
AspAp	0.3673	0.193	0.037
GluAp	0.3843	0.186	0.035
NGAL	0.6847	−0.087	0.008
Proteinuria	0.8324	0.046	0.002
NAG	0.9255	−0.020	0.000
Plasma creatinine			
AlaAp*	0.0053	0.550	0.303
CysAp	0.0561	0.395	0.156
Albumin	0.0607	0.388	0.151
AspAp	0.1650	0.293	0.086
GluAp	0.2437	0.247	0.061
NAG	0.2511	−0.244	0.059
Proteinuria	0.8290	−0.047	0.217
NGAL	0.8905	−0.030	0.088
Creatinine clearance			
AlaAp*	0.0001	−0.712	0.507
CysAp*	0.0015	−0.611	0.374
GluAp	0.0142	−0.494	0.244
AspAp	0.0158	−0.487	0.237
Albumin	0.1036	−0.340	0.116
Proteinuria	0.4234	−0.174	0.029
NGAL	0.7485	0.069	0.005
NAG	0.9409	0.016	0.000

*$p<0.05$ and $|r| > 0.5$.
Biomarkers are rank ordered for p-value from top to bottom.
doi:10.1371/journal.pone.0040402.t002

Results

Figure 1 shows the time course of aminopeptidase urinary excretion in experimental groups. Ala, Glu, Cys and AspAp activities were increased at day 1, 2 and 3 after treatment in CisPt7 group. We also found a significantly increase of CysAp activity at day 2 in CisPt3.5 group (Fig. 1C).

Albuminuria was increased at day 1, 2 and 3 in CisPt7 group, while proteinuria, NAG and NGAL were increased at day 3 (Fig. 2). Albuminuria and NAG increased at day 2 and 3 in CisPt 3.5 group. Proteinuria and NGAL excretion were also significatively augmented at day 3 in CisPt 3.5 group.

At the end of the experiment, CisPt7 group exhibited an augmented plasma creatinine level, diminished creatinine clearance and renal hypertrophy (Fig. 3), while CisPt3.5 group did not show any statistically significative difference in these parameters with respect to control group.

The renal lesions in Wistar control rats were absent. No glomerular, tubulointerstitial or vascular lesions were present in renal parenchyma. Histopathological examination of renal slices from rats treated with 3.5 or 7 mg/kg of cisplatin showed different alterations, including relevant nuclear dysplasia and incipient acute tubular necrosis (Fig. 4). The sections of the kidney from cisplatin-treated rats exhibited marked dilation of proximal convoluted tubules in the corticomedullary junction with slogging of almost entire epithelium due to desquamation of tubular epithelium that induced a total absence of microvilli and loss of brush border in CisPt7 group. Also, mild tubular atrophy and apoptotic cells in tubular lumen were present in both cisplatin-treated groups (Table 1). Statistical differences were found between both cisplatin groups into morphological variables analysis, indicating a dose-response relationship.

Cisplatin significantly enlarged the fibrosis area in the tubular interstitium from CisPt3.5 and CisPt7 groups. The fibrosis area was remarkably higher in the corticomedullary junction of the kidney (Table 1, Fig. 4).

Significative correlations were found for Ala, Glu, Cys and AspAp urinary activities at 24 hours of treatment with kidney weight/body weight ratio at the end of the experiment. AlaAp and CysAp activities correlated with creatinine clearance, and AlaAp also correlated with plasma creatinine (Table 2, Fig. 5). We did not found a correlation of any of biomarkers studied at this point with renal fibrosis (Table 2).

AlaAp presented the largest ROC-AUC of all the markers studied 24 hours after treatment (Fig. 6). Therefore, AlaAp showed the maximum levels of specificity and sensitivity to detect if an animal received cisplatin or saline and it was a perfect discriminator between treated and untreated rats. GluAp, CysAp and AspAp also had an AUC >0.5, as albuminuria and proteinuria (Table 3), indicating that these enzymes could also be considerated as biomarkers of renal dysfunction in this animal model.

Discussion

The main finding of this article is that Ala, Glu, Cys, and AspAp activities are early and predictive urinary biomarkers of the AKI induced by cisplatin, and their determination could be very useful in early detection and monitorization of renal damage.

Biochemical and histhopathological data obtained in our experimental groups were in consonance with those obtained in previous works by other authors [14,15] and confirmed the effectiveness of cisplatin treatment producing renal injury, showing similar increases in plasmatic creatinine, proteinuria and enzymuria at the same days of the study.

Ala, Glu and CysAp are highly organ-specific because they are present in the brush border membrane of renal tubular cells [9] and exhibit high molecular weights (>140 kDa) that make difficult that their presence in urine could be due to alterations in glomerular barrier. These enzymes participate in the intrarrenal renin-angiotensin-aldosterone system (RAAS) [20]. It is known that increased tubular absorption of filtered proteins induces tubulointerstitial inflammation, ultimately resulting in tubular

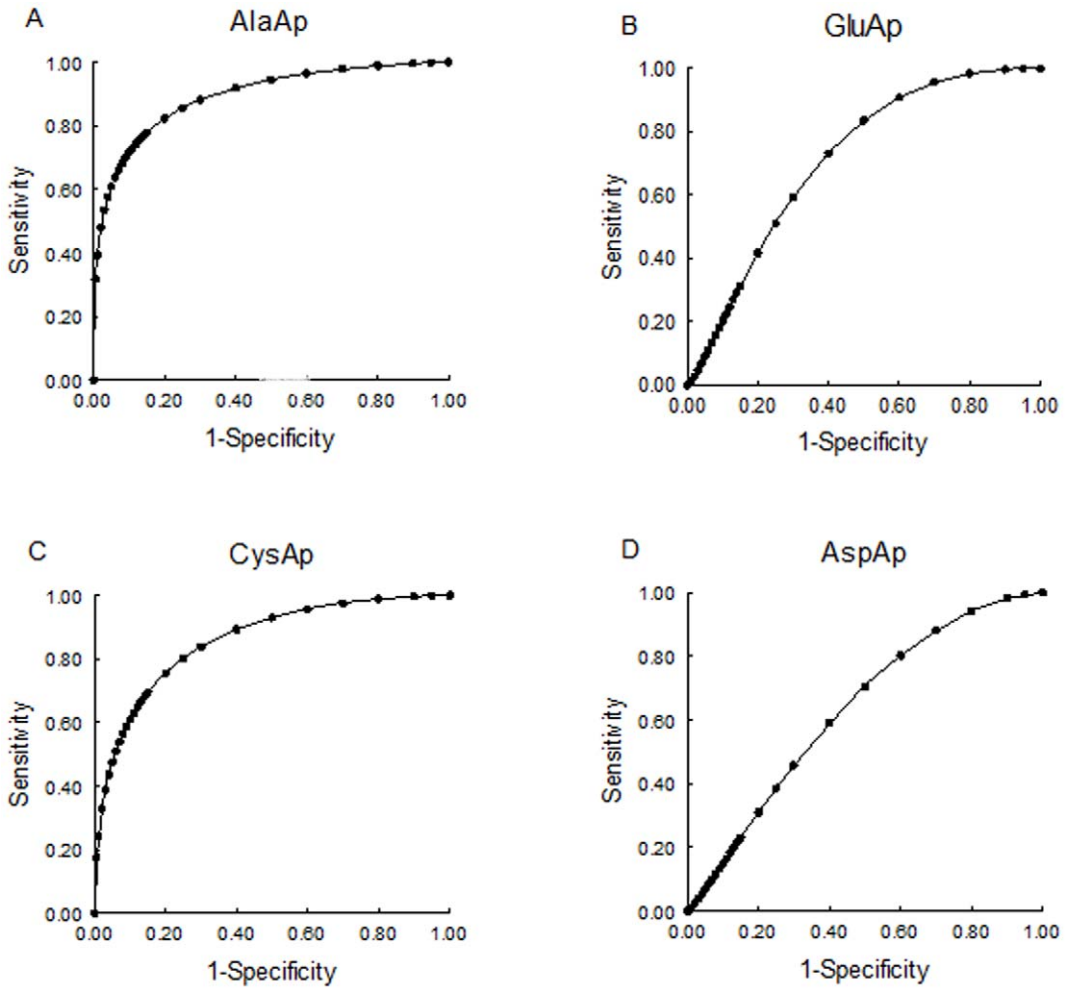

Figure 6. ROC curves for urinary aminopeptidase activities at day 1. ROC curves showing specificity and sensitivity for AlaAp (A), GluAp (B), CysAp (C) and AspAp (D) urinary activities at the first day of treatment.
doi:10.1371/journal.pone.0040402.g006

atrophy, interstitial fibrosis, and loss of renal function [21]. In the proximal tubule, albumin and other ultrafiltered proteins are reabsorbed by endocytosis involving megalin and cubulin [22]. AngII stimulates albumin endocytosis in proximal tubule cells *via* AT2 receptor–mediated protein kinase B activation. However, an increase in tubular albumin reabsorption activates the tubular RAAS, leading to a vicious circle [23] that could explain the elevated urinary activities of these enzymes when they are released from the brush border to the ultrafiltrate after cisplatin injection.

Table 3. ROC-Area under the curve (AUC), sensitivity at 95% specificity (Sens 95%) and fold-cutoff relative to controls to achieve 95% specificity (threshold) of urinary biomarkers excreted at day 1 in control (true negative) and cisplatin 3.5 or 7 mg/kg (true positive) treated rats.

	AUC	Sens 95%	Threshold
AlaAp	0.894±0.067	0.6090	1.7394
CysAp	0.860±0.079	0.4757	1.6209
Albumin	0.806±0.087	0.5321	1.7196
GluAp	0.714±0.119	0.0895	1.7079
Proteinuria	0.709±0.114	0.1354	1.6543
AspAp	0.633±0.125	0.0683	1.6299
NAG	0.453±0.114	0.1492	1.5392
NGAL	0.377±0.124	0.0129	1.7247

Biomarkers are rank ordered for AUC from top to bottom.
doi:10.1371/journal.pone.0040402.t003

In consequence, AlaAp activity has been widely used as a marker of renal dysfunction in animal models of nephrotoxicity induced with vancomicine [24] or amphotericin B [25], or in human diseases like glomerulopathies [26], IgA nephropathy [27], rheumatoid arthritis [28] or diabetes [29], and there are contradictory results about its utility as a marker in kidney transplanted patients [30,31].

The high correlation of urinary activities of Glu, Ala, Cys and AspAp at day 1 with kidney weight/body weight ratio, and of Ala and CysAp with creatinine clearance at day 14 shows their predictive value over renal dysfunction and structural damage. AlaAp also correlates with plasma creatinine at day 14. Nevertheless, there is no correlation in any of the biomarkers studied with renal fibrosis. Kawai et al [15] concluded that fibrosis is a process of resolution from acute tubular injury induced by cisplatin and, in our study, we also have found an increase in the area of interstitial fibrosis at day 14 in the two groups of animals treated

with cisplatin. The lack of correlation of all urinary biomarkers with renal fibrosis may indicate that the extent of fibrotic lesions is not directly related with the tubular injury evoked by cisplatin at 24 hours. In fact, the group treated with the submaximal dose of cisplatin showed a fibrosis area similar to the group treated with 7 mg/kg. Nevertheless, the CisPt3.5 group had unaltered kidney weight/body weight, plasma creatinine or creatinine clearance. This would indicate that the alterations in renal function observed in CisPt7 group are more dependent of other factors like tubular dilation or dysplasia rather than of the fibrotic process.

Interestingly, the rest of the markers studied did not correlate with any parameter of renal dysfunction or structural damage, and albuminuria was the only marker that was slightly increased at 24 hours in CisPt7 group. Low-molecular weight proteins like albumin can pass through glomerular barrier and, therefore, urinary proteins and albumin may have an extrarrenal origin in other patologies. In a previous study, albuminuria has been related with cardiovascular events like endothelial injury that presents collateral kidney damage [32]. Therefore, the urinary levels of albumin or total proteins may be influenced by their plasmatic concentration, making difficult to elucidate in some cases if their presence in urine is exclusively due to the renal alterations. In our experiment, the slight elevation of albuminuria at 24 hours in CisPt7 group may be probably related with the reduction in the tubular reabsorption of this protein due to the release of aminopeptidases from microvilli, because these enzymes were the only markers that were increased at this point.

24 hours after treatment there were no differences in the excretion of NAG and NGAL in the animals treated with the submaximal or the maximal dose of cisplatin *versus* control group. Therefore, in this model, NAG and NGAL could not detect the renal damage evoked with cisplatin as early as aminopeptidase activities or albuminuria and they do not predict renal alterations 24 hours after treatment as Ala, Glu, Cys or AspAp activities do. NAG and NGAL are implicated in several tubular functions that include transport of proteins [16] and hydrophobic molecules [33] through membranes, respectively. Our findings suggest that the release of aminopeptidases from brush border precedes in time to the alterations in these renal tubular functions in this model. NAG and albuminuria started to increase at day 2 in CisPt3.5 group, and NGAL and proteinuria at day 3, while aminopeptidase activities were not significatively increased, except a slight increase in CysAp activity at day 2. This would indicate that the submaximal dose of cisplatin evokes alterations in renal tubular transport functions that are similar to the alterations observed with the maximal dose of cisplatin. In fact, there were no differences in the excretion of protein, albumin, NAG and NGAL between CisPt3.5 and CisPt7 group. Nevertheless, this tubular dysfunction is not accompanied with a significative loss of brush border enzymes or, at least, it is not sufficiently remarkable with this submaximal dose.

In conclusion, Ala, Glu, Cys and AspAp enzymatic activities are early and predictive biomarkers of the AKI induced by cisplatin. The four markers show high sensitivity and specificity to distinguish nephrotoxicant-treated from control rats. These determinations could be very useful in the prognostic and diagnostic of renal dysfunction in preclinical research and clinical practice, because the urine sample does not need any special treatment and the laboratory test is simple, quick and inexpensive in comparison with other techniques that require expensive antibodies and large periods of incubation, like ELISA or Western blot, that sometimes make difficult their serial determination.

Author Contributions

Conceived and designed the experiments: AQ FV RW AO. Performed the experiments: AQ SMM FO MDRM IP RW. Analyzed the data: AQ SMM FO MDRM RW AO. Contributed reagents/materials/analysis tools: FO IP MR FV RW. Wrote the paper: AQ SMM FO MR FV RW.

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