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Maestría y Doctorado en Ciencias Bioquímicas

**EFEECTO DE LA FICOCIANINA SOBRE EL DAÑO
INDUCIDO POR CISPLATINO: ESTUDIOS *IN VITRO* E *IN
VIVO***

TESIS

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ABREVIATURAS

$^1\text{O}_2$	Singulete de oxígeno
4-HNE	4-hidroxi-2-nonenal
$\cdot\text{OH}$	Radical hidroxilo
$\cdot\text{NO}$	Óxido nítrico
A	Absorbancia
ADN	Ácido desoxirribonucleico
ADP	Adenosin difosfato
ADP/O	Cociente de la fosforilación de ADP por mol de oxígeno
ANOVA	Análisis de varianza
ASB	Albúmina sérica bovina
ATP	Adenosin trifosfato
BUN	Nitrógeno de urea en sangre
Ca^{2+}	Ión calcio
CAT	Catalasa
CI_{50}	Concentración inhibitoria 50
CP	Cisplatino
CsA	Ciclosporina
CTE	Cadena de transporte de electrones
CTR-1	Transportador de cobre
DMEM	Medio Dulbecco's Modified Eagle
E.E.	Error estándar
ERO	Especie reactiva de oxígeno
FC	Ficocianina
FCB	Ficocianobilina
FCCP	Carbonilcianuro-p-trifluorometoxi-hidrazona
FDA	Food and Drug Administration
G6PD	Glucosa 6-fosfato deshidrogenasa
GPx	Glutación peroxidasa
GRAS	Generalmente reconocido como seguro
GSH	Glutación reducido
GSSG	Glutación oxidado
GST	Glutación S-transferasa
H&E	Hematoxilina y eosina
H_2O_2	Peróxido de hidrógeno
i.p.	Intraperitoneal
ICR	Índice de coeficiente respiratorio
LLC-PK1	Células de túbulo proximal de cerdo
MDA	Malondialdehído
MME	Membrana mitocondrial externa
MMI	Membrana mitocondrial interna
NADH	Nicotinamida adenina dinucleótido

NADPH	Nicotinamida adenina dinucleótido fosfato (reducido)
NAG	N-acetil- β -D-glucosaminidasa
NBT	Azul de tetrazolio
NOAEL	Nivel sin efecto adverso observado
O	Oxígeno molecular
O ₂ ^{•-}	Radical anión superóxido
OCT-2	Transportador de cationes orgánicos
ONOO ⁻	Anión peroxinitrito
PTPm	Poros de transición de la permeabilidad mitocondrial
RO [•]	Alcoxilo
ROO [•]	Peroxilo
T	Testigo

RESUMEN

Antecedentes: La ficocianina (FC) es un nutraceutico antioxidante de origen proteico empleado en la formulación de diversos alimentos. Posee diversas propiedades, entre ellas la de anti-oxidante. Por otra parte, el cisplatino (CP) es un antineoplásico empleado en todo el mundo para el tratamiento de tumores, desafortunadamente induce daño renal en un 30% de los pacientes. Uno de los mecanismos involucrados en el daño renal es el estrés oxidante, por lo tanto el consumo de antioxidantes puede ser relevante para atenuar este daño.

Justificación: El CP es un fármaco eficaz en el tratamiento de tumores sólidos. Sin embargo, su efecto secundario más importante es la nefrotoxicidad relacionada con el estrés oxidante. De esta manera y considerando que la FC es un nutraceutico con capacidad antioxidante, se propone que este compuesto puede reducir los efectos nefrotóxicos del CP. Nuestro estudio contribuirá a la búsqueda de compuestos de origen natural que puedan emplearse para prevenir el daño renal inducido por CP.

Hipótesis: Si la FC atenúa el estrés oxidante y las alteraciones mitocondriales, entonces su administración reducirá la nefrotoxicidad inducida por CP en ratones CD-1.

Objetivo general: Determinar si el nutraceutico FC protege contra el daño renal inducido por cisplatino en ratones CD-1 e investigar el mecanismo de acción mediante la determinación de enzimas antioxidantes, el daño oxidante y la función mitocondrial.

Resultados: La FC empleada presentó actividad antioxidante *in vitro* contra peroxinitrito, oxígeno singulete, peroxilo, hidroxilo, superóxido y peróxido de hidrógeno. En ratones CD-1, la FC previno el daño renal por CP de manera dosis-dependiente (5-30 mg/kg i.p.). Dicho daño se evaluó mediante la medición de creatinina, nitrógeno de urea plasmática (BUN) y por estudios histológicos. La FC

redujo los marcadores de estrés oxidante empleados (contenido renal de malondialdehído, 4-hidroxinonenal y proteínas oxidadas). Se encontró que la FC previno la disminución de la actividad de enzimas antioxidantes: glutatión reductasa (GR), glutatión-S-transferasa (GST) y catalasa (CAT). Los datos de oximetría de los grupos: testigo, FC (30 mg/kg i.p.), CP (22 mg/kg i.p.) y FC+CP revelaron que la FC previene la disfunción mitocondrial evaluada tanto con malato:glutamato como con succinato, como sustratos; atenuó la reducción del índice del coeficiente respiratorio (ICR). El pre-tratamiento con FC previno la pérdida del potencial de membrana, la reducción del manejo de calcio mitocondrial, redujo el daño oxidante (actividad de aconitasa, contenido de glutatión reducido), además previene la reducción de la actividad de enzimas antioxidantes mitocondriales (GPx y CAT).

Conclusión: El nutracéutico FC previene el daño renal y la disfunción mitocondrial inducidos por CP en ratones CD-1. El efecto protector de la FC en el modelo *in vivo*, se asoció con la actividad antioxidante de la FC contra las especies inducidas por CP. La protección de la FC en los ratones CD-1 se asoció con la atenuación del estrés oxidante y la prevención en la pérdida de la actividad antioxidante tanto en el riñón como en las mitocondrias renales.

1 INTRODUCCIÓN

1.1 Alimentos funcionales

En diversos estudios se ha demostrado la relación entre los alimentos y la salud (Jones y Varady, 2008; Rudkowska y Jones, 2007). De este modo se ha observado un interés mayor en el área de interacción alimentos-salud o de los alimentos funcionales y la búsqueda de productos naturales lo que lleva a altas ventas a nivel internacional (Bech-Larsen y Scholderer, 2007).

La investigación de estos alimentos surgió en Japón, país que sigue siendo el principal innovador. El término alimento funcional define a un grupo de alimentos con propiedades benéficas para la salud que van más allá de las claramente adscritas a los nutrientes que contienen (Quinna *et al.*, 2013; Ferguson, 2009; Jones y Varady, 2008).

Éstos tienen la característica general de contener nutraceuticos, término que resulta de la combinación de nutrición y farmacéutico, son sustancias naturales bioactivas presentes usualmente en los alimentos y que tomadas en dosis superiores a la existente en ellos, tienen un efecto favorable sobre la salud (Gil-Chávez *et al.*, 2013).

Actualmente es aceptado el papel de los nutraceuticos en la prevención, tratamiento y/o reducción del riesgo de padecer algún tipo de enfermedad (Lobo *et al.*, 2010; Schroeder, 2007). Un grupo de estos nutraceuticos son los antioxidantes.

1.2 Antioxidantes y especies reactivas de oxígeno

Un antioxidante retrasa o inhibe significativamente la oxidación de moléculas (González *et al.*, 2009). Uno de los mecanismos de acción de estos compuestos es aquél en que la molécula antioxidante, al reaccionar con un radical libre de oxígeno o de nitrógeno, le cede un electrón, que se oxida a su vez y se transforma en una molécula o radical libre menos reactivo.

Es importante mencionar que dichos compuestos pueden adquirirse a través de la dieta o sintetizarse *in vivo*. Estos últimos son proteínas que remueven especies reactivas de oxígeno (ERO) y otras especies, como es el caso de las enzimas antioxidantes como superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx), etc.

Las ERO son compuestos, que pueden o no ser radicales libres en las cuales el oxígeno está parcialmente reducido (Halliwell y Gutteridge, 2010).

Por otra parte, un radical libre es cualquier especie que contiene uno o más electrones desapareados dentro de su estructura (Halliwell y Gutteridge, 2010). Dichos compuestos se representan colocando un punto sobre el símbolo del átomo que contiene el electrón desapareado y generalmente, son sustancias altamente reactivas que intentan completar sus electrones tomándolos de otros compuestos, causando su oxidación. Por lo anterior, se les denomina oxidantes (Ďuračková, 2010; Konigsberg, 2008).

En la reducción univalente del oxígeno, las ERO se forman con la adición de un electrón a una molécula de oxígeno produciendo a su vez especies parcialmente reducidas las cuales pueden reaccionar para producir otras ERO (Figura 1).

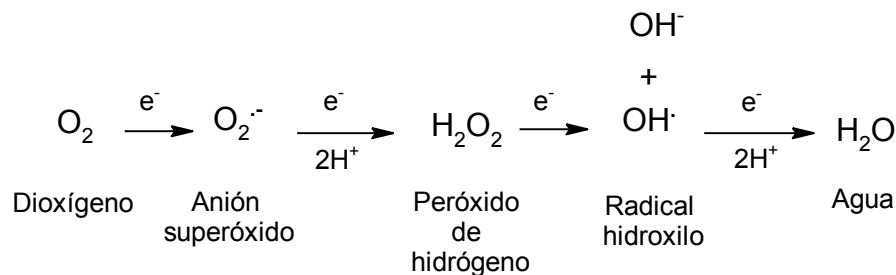


Figura 1. Formación de ERO por la reducción univalente del oxígeno (tomado de Halliwell y Gutteridge, 2010).

Es importante mencionar que las ERO en concentraciones bajas tienden a estimular el crecimiento de las células, funcionan como señales que determinan respuestas fisiológicas de adaptación al medio ambiente y son indispensables

para la diferenciación celular y para la muerte celular programada (Konigsberg, 2008). Sin embargo, cuando se encuentran en exceso, tienen la característica de reaccionar con otros radicales o con biomoléculas de gran importancia biológica, como ADN, proteínas y lípidos que promueven un daño fisiológico relevante, es decir, provocan daño oxidante.

Los radicales pueden generarse de manera endógena y exógena. Las ERO endógenas son producidas principalmente por la mitocondria durante la fosforilación oxidativa, citocromo P450, procesos inflamatorios, etc. Por otra parte, las ERO exógenas provienen de la contaminación ambiental. Por lo tanto, es importante el consumo de alimentos que contengan antioxidantes para mantener el equilibrio entre radicales y antioxidantes. Se ha observado que el consumo de alimentos que contienen antioxidantes se relaciona con una mejoría en diversas patologías (Montagna *et al.*, 2005; Cermak, 2008).

1.3 Ficocianina

Es uno de los principales pigmentos del alga de origen mexicano espirulina, la cual es empleada como suplemento alimenticio debido a su alto contenido de proteínas, vitaminas, minerales y ácidos grasos esenciales (Habib *et al.*, 2008). La ficocianina (FC) se encuentra tanto en algas de cianobacterias como en células eucariontes como *Rhodophyta*. En ambas, proporciona un color característico verde-azul (Eriksen, 2008).

La FC es un compuesto de color azul intenso de origen proteico que forma parte de las ficobiliproteínas. Es un pigmento antena recolector de luz organizado en complejos supramoleculares denominados ficobilosomas. Los ficobilosomas se encuentran ensamblados en la superficie de la membrana tilacoidal, su principal función es transferir la energía de excitación hacia el centro de reacción. Su longitud de onda máxima de absorción es de 620 nm.

La pureza de la FC es evaluada mediante el cociente de absorbancias A620/A280. La absorbancia a 620 y 280 nm corresponden a la FC y a las proteínas totales, respectivamente (Patil *et al.*, 2006). La FC grado alimenticio corresponde al cociente $A_{620}/A_{280} \leq 0.7$, el grado reactivo cuando A_{620}/A_{280} está entre 0.7 y 3.9 y el grado analítico es $A_{620}/A_{280} \geq 4.0$ (Antelo *et al.*, 2010; Kuddus *et al.*, 2013; Patil *et al.*, 2006).

La FC grado alimenticio es un alimento generalmente reconocido como seguro (GRAS) y se emplea en la formulación de diversos productos alimenticios como colorante en bebidas alcohólicas, postres, dulces, decoración de pasteles, batidos de leche, cosméticos, biotecnología, diagnóstico y en la medicina (Kim *et al.*, 2008; Yoshida *et al.*, 1996).

La dosis más alta utilizada con el nivel sin efecto adverso observado (NOAEL) fue estudiado por Romay *et al.* (1998b) quienes encontraron un NOAEL (por vía oral) de 3 g/kg. Actualmente se ha demostrado que la FC administrado por vía oral tiene un NOAEL de 5 g/kg (Ou *et al.*, 2012). Por otra parte, el NOAEL de 70 mg/kg por vía intraperitoneal (i.p.) se utilizó en ratas, sin mostrar alteraciones en comparación al grupo testigo (Gupta *et al.*, 2011). Sin embargo, la dosis más alta de 200 mg/kg i.p. se ha utilizado en experimentos con ratas Wistar sin observar alteraciones hepáticas (González *et al.*, 2003; Vadiraja *et al.*, 1998).

1.3.1 Estructura de la FC

La FC está formada de dos subunidades homólogas, las cadenas α y β unidas covalentemente a la ficocianobilina (FCB) mediante un enlace tioeter (Figura 2). Éstos forman agregados naturalmente como trímero ($\alpha_3\beta_3$) o hexámero ($\alpha_6\beta_6$), éste último es la forma funcional de la FC y por lo general forma bloques con el complejo antena (Padyana *et al.*, 2001; Scheer y Zhao, 2008). La $\alpha_3\beta_3$ se compone de nueve unidades de PCB montados en tres partes iguales (Womick y Moran, 2009).

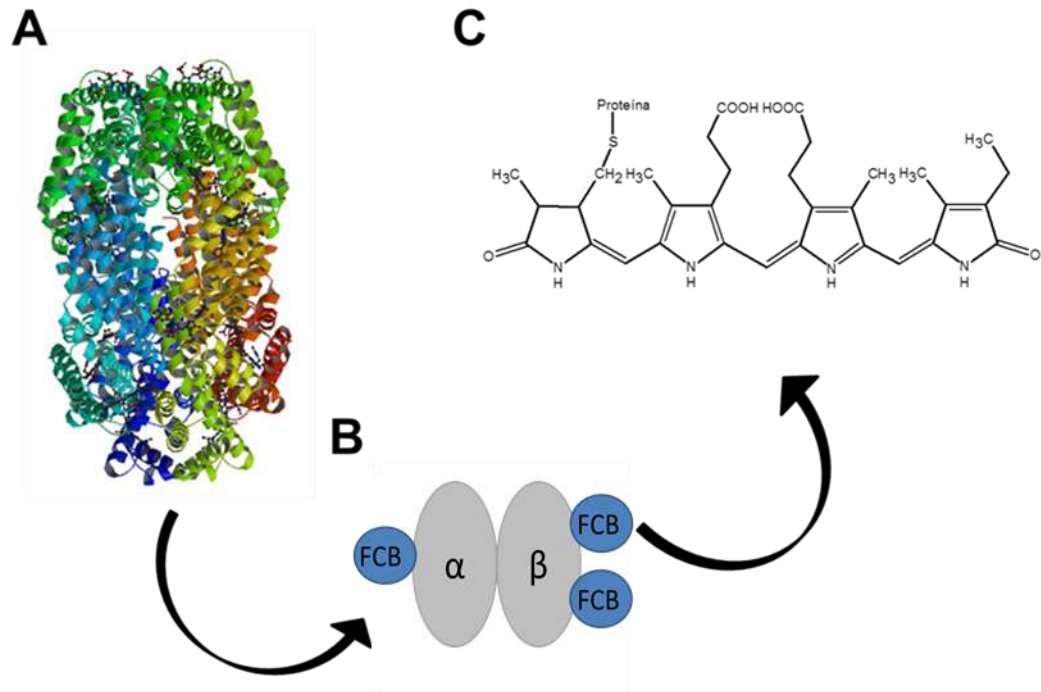


Figura 2. Estructura de la ficocianina (FC). **A:** Estructura cristalina de FC de la cianobacteria *S. platensis* en forma de hexámero, imagen tomada de AP ID 1GH0 (www.rcsb.org). **B:** Representación del ensamblaje de FC. Se compone de dos subunidades proteicas, las subunidades α y β , una ficocianobilina (FCB) que está unida a la subunidad α y dos FCB que están unidas a la subunidad β . **C:** Estructura química de FCB, el cromógeno responsable del color azul de la FC (Fernández-Rojas *et al.*, 2014a).

El peso molecular de las subunidades varía dependiendo de la fuente y los métodos para la extracción y purificación de las algas. El peso molecular de la subunidad α está entre 13 y 20,5 kDa y la subunidad β está entre 11 y 24,4 kDa (Fernández-Rojas *et al.*, 2014a).

La FCB posee una estructura muy similar a la bilirrubina, de hecho, se sugiere que el mecanismo de acción es mimetizar a la biliverdina (Eriksen, 2008). Es un antioxidante capaz de neutralizar diversas ERO como se menciona en el siguiente apartado.

1.4 La ficocianina como antioxidante

La primera investigación de la actividad antioxidante de FC se realizó por investigadores Cubanos en el año de 1998 (Romay *et al.*, 1998b) quienes determinaron que es capaz de neutralizar los radicales libres hidroxilo ($\cdot\text{OH}$), alcoxilo ($\text{RO}\cdot$) y superóxido ($\text{O}_2^{\cdot-}$) así como de inhibir la lipoperoxidación, incluso sentaron las bases de la propiedad anti-inflamatoria. Posteriormente, otros investigadores de varias partes del mundo descubrieran que también neutraliza a las ERO singulete de oxígeno ($^1\text{O}_2$) (Tapia *et al.*, 1999), radical peroxilo ($\text{ROO}\cdot$) (Benedetti *et al.*, 2010; Romay *et al.*, 2000), anión peroxinitrito (ONOO^-) (Bhat y Madyastha, 2001), óxido nítrico ($\cdot\text{NO}$) (Thangam *et al.*, 2013) y al peróxido de hidrógeno (H_2O_2) (Fernández-Rojas *et al.*, 2014b) (Tabla 1).

Durante dicho proceso el color de la FC se degrada, reduce gradualmente su absorbancia a 620 nm y su intensidad fluorométrica (Patel *et al.*, 2005; Benedetti *et al.*, 2004; Lissi *et al.*, 2000; Tapia *et al.*, 1999).

Tabla 1. Concentración inhibitoria del 50% (CI_{50}) de FC contra diversas ERO.

Radical	CI_{50}	Referencia
$\text{RO}\cdot$ (Alcoxilo)	0.076 mg/mL	Romay <i>et al.</i> (1998b)
$\text{ROO}\cdot$ (Peroxilo)	0.170 mg/mL	Bhat y Madyastha (2000)
$\text{O}_2^{\cdot-}$ (Anión superóxido)	0.440 mg/mL	Chen y Wong (2008)
ONOO^- (Peroxinitrito)	0.810 mg/mL	Bhat y Madyastha (2001)
$\text{OH}\cdot$ (Hidroxilo)	0.910 mg/mL	Romay <i>et al.</i> (1998b)

Actualmente, es empleado como un agente protector ante diversos retos oxidantes. Se ha establecido que posee varias propiedades benéficas ya que es capaz de prevenir diversas enfermedades relacionadas con el estrés oxidante (Fernández-Rojas *et al.*, 2014a).

1.5 Cisplatino

El cisplatino o cis-diaminodicloroplatino (CP, cis-PtCl₂(NH₃)₂) es un antineoplásico inorgánico neutro que se emplea en la clínica para el tratamiento de tumores sólidos. Está formado por un átomo de platino en el centro de la estructura, al cual están unidos dos átomos de cloro y dos grupos amino (Figura 3). Fue sintetizado por M. Peyrone en 1844 y su estructura química fue dilucidada por Alfred Werner en 1893 (Dasari y Tchounwu, 2014). Sin embargo; el compuesto permaneció sin ser investigado varios años, hasta la década de los 60's, cuando fue estudiado por el equipo de Rosenberg, quienes demostraron que determinados productos de electrólisis con electrodos de platino, inhibían la división celular de *Escherichia coli* (Rosenberg, 1985). Tras la identificación de CP como el compuesto responsable de la actividad inhibitoria de la división celular, éste se ha empleado en el tratamiento de tumores. Posteriormente en 1978 la FDA (del inglés Food and Drug Administration) aprobó el uso del CP como un antineoplásico (Lebwohl y Canetta, 1998).

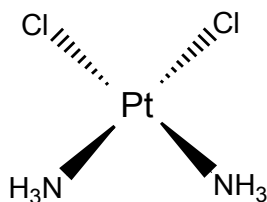


Figura 3. Molécula de cisplatino.

Actualmente, el CP es uno de los fármacos más utilizados (Santana *et al.*, 2010) y presenta excelentes efectos terapéuticos en el tratamiento de tumores sólidos como los de pulmón, ovario, testículo, vejiga, cabeza, cuello, cervix y endometrio (Sánchez-Pérez *et al.*, 2010). Sin embargo; éste presenta efectos secundarios significativos como náuseas, vómito, nefrotoxicidad, problemas gastrointestinales y frecuencia alta de la pérdida auditiva.

En el plasma, una parte del CP se une a proteínas plasmáticas y otra entra a las células por transportadores catiónicos orgánicos (OCT-2) (Dasari y Tchounwu,

2014). En la célula, se lleva a cabo un desplazamiento de las moléculas del cloro del CP por agua, lo que evita que pueda salir de la célula (Figura 4). La especie cargada del CP es un electrófilo potente que puede reaccionar con nucleófilos con grupos sulfhidrilos de proteínas o nitrógenos del ADN. El CP se une al ADN en las bases que poseen pares de electrones libres; sobre todo al nitrógeno en posición 7 de la guanina. El CP forma aductos adenina-guanina o guanina-guanina intracatenaria o intercatenaria llevando a la célula a muerte celular (Nakai *et al.*, 2011; Chirino y Pedraza-Chaverri, 2009). Sin embargo, la acción del CP no es específica de células tumorales y por consiguiente puede ejercer sus efectos adversos en otras células (Ruiz *et al.*, 2011).

Este fármaco se metaboliza y se excreta principalmente por vía renal, órgano donde se acumula y ejerce sus efectos adversos. Las células más afectadas son las del segmento S3 del túbulo proximal (Hausheer *et al.*, 2011) y esta alteración puede causar disfunción renal limitando aún más la eliminación del CP.

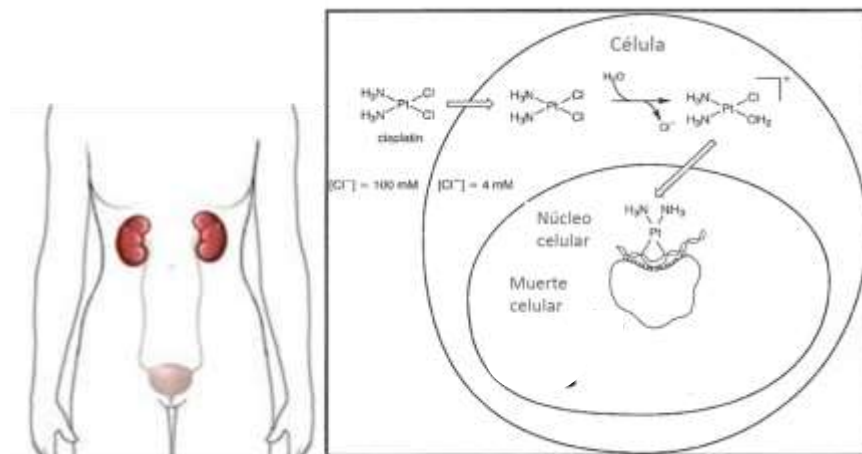


Figura 4. Efecto citotóxico del CP. Cuando el cisplatino entra a la célula se desplazan los cloros por agua, quedando así activo e intercalándose en el ADN induciendo la muerte celular, el órgano en donde se concentra es el riñón; así induce daño renal.

En clínica, hasta un 30% de los pacientes expuestos a tratamiento con CP presenta toxicidad renal (dos Santos *et al.*, 2012). Esto ha limitado el uso del CP como agente quimioterapéutico (Gonzalez-Vitale *et al.*, 1977). Generalmente,

después de 10 días de su administración, los pacientes presentan un incremento de los niveles séricos de creatinina y disminución de la filtración glomerular además se observa necrosis y apoptosis en estudios histológicos (Hassan *et al.*, 2010; Gonzales-Vitale *et al.*, 1977).

Patológicamente, induce lesión celular y la muerte en los túbulos renales, desencadena disfunción vascular y activa una respuesta inflamatoria

1.6 Estrés oxidante inducido por CP

El estrés oxidante se define como el desequilibrio entre la generación de ERO y las defensas antioxidantes (Halliwell, 2012; Silva y Coutinho, 2010). Este desequilibrio puede ser generado por una producción excesiva de ERO, por una disminución de las defensas antioxidantes o por ambos casos. Bajo condiciones de estrés oxidante, se conduce al daño a proteínas, lípidos y ADN (Gülçin, 2012; Halliwell, 1996).

Aunque los mecanismos celulares y moleculares responsables de la toxicidad del CP no están bien establecidos, se ha demostrado que la formación de ERO, la reducción de enzimas antioxidantes y el daño mitocondrial están involucrados (Rodrigues *et al.*, 2011). La comprensión de este mecanismo podría dar pie a la intervención de protectores novedosos (El-Sayed *et al.*, 2011). Por esta razón varios investigadores se han dado a la búsqueda de productos que reduzcan estos daños. Incluso existe evidencia que la administración de antioxidantes pudiera reducir la toxicidad inducida por CP (El-Sayed *et al.*, 2011). En este punto, se ha identificado a la disfunción mitocondrial (incremento de ERO) como un posible mecanismo de daño renal inducido por CP (Chang *et al.*, 2002; Li-Ping *et al.*, 2000).

1.7 Características estructurales de mitocondrias y las tratadas con CP

Las mitocondrias son orgánulos presentes en las células eucariotas, varían de tamaño y forma, dependiendo de su origen y su estado metabólico (Lehninger, 1995). Poseen dos membranas, una membrana mitocondrial externa (MME) que

envuelve completamente a la membrana mitocondrial interna (MMI) y forma un espacio intermembrana (Figura 5). La MME tiene poros que permite el paso de iones y moléculas pequeñas. En contraste, la MMI es selectivamente permeable, es impermeable a iones y moléculas pequeñas, permite el paso de moléculas por medio de un transportador específico para éstas. Contiene las proteínas implicadas en el transporte de electrones y la síntesis de ATP. Además, tiene pliegues internos o invaginaciones llamadas crestas. Las crestas aumentan el área superficial de la MMI en relación con la matriz mitocondrial (Che *et al.*, 2014). Esta membrana envuelve a la matriz mitocondrial.

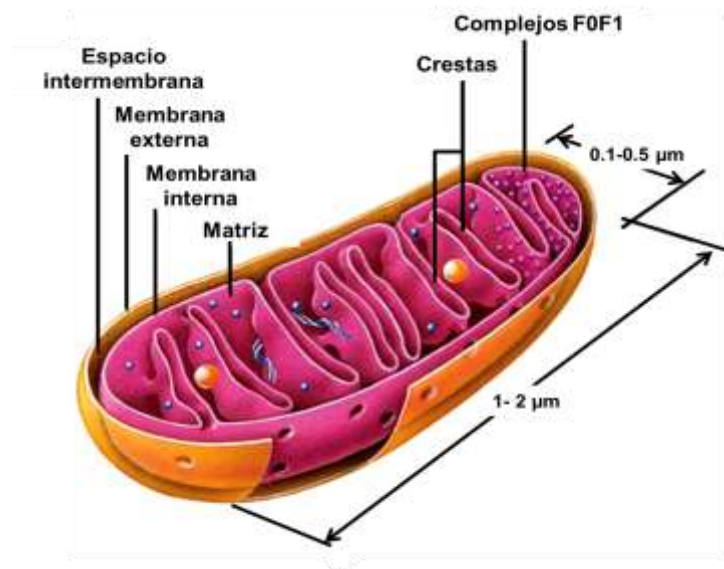


Figura 5. Estructura mitocondrial. Tomado de Lodish *et al.* (2005).

Los cambios patológicos en las mitocondrias en la nefrotoxicidad de cisplatino son provocados principalmente por la respuesta de daño en el DNA, la inducción de proteínas pro-apoptóticas, la alteración de la dinámica mitocondrial, la inducción de apoptosis por la vía intrínseca y el estrés oxidativo (Yang *et al.*, 2014).

Se ha descrito que las mitocondrias aisladas de roedores tratados con cisplatino poseen daño estructural. Estas mitocondrias presentan rigidez de la membrana mitocondrial, alteración de la fluidez de la misma, hinchamiento, incremento del área de la matriz mitocondrial, alteración de las crestas incluso algunas

mitocondrias pierden la membrana externa (Zsengellér *et al.*, 2012; Martins *et al.*, 2008; Gordon y Gattone, 1986). El conjunto de estas alteraciones puede ser fatal para las células.

1.8 Cadena de transporte de electrones y fosforilación oxidativa

La cadena de transporte de electrones (CTE) es el flujo de electrones a complejos proteicos, conocidos como complejos mitocondriales. Los electrones son transportados del complejo I (NADH deshidrogenasa) y II (succinato deshidrogenasa) al complejo III (citocromo c oxidorreductasa) mediante la ubiquinona y desde el complejo III al complejo IV (citocromo c oxidasa) mediante el citocromo c (Figura 6). Estos complejos se describen brevemente a continuación.

Complejo I o NADH deshidrogenasa. Es el complejo más grande de la CTE formada por 45 subunidades. Contiene un grupo flavin mono nucleótido (FMN) y al menos 6 centros hierro azufre (Fe-S). Este complejo cataliza la transferencia de un par de electrones del NADH a la ubiquinona, acoplada a la traslocación de cuatro protones de la matriz al espacio intermembranal. Su estructura tiene forma de L con dos brazos, uno de ellos se encuentra embebido en la MMI y el otro está orientado hacia la matriz mitocondrial (Pagliarini y Rutter, 2013).

Complejo II o succinato deshidrogenasa. Está formado por 4 subunidades, dos subunidades son membranales y las otras dos están orientadas hacia la matriz mitocondrial. El primer aceptor de electrones es el flavín adenín dinucleótido (FAD), posteriormente son transferidos por varios centros Fe-S y finalmente la ubiquinona es reducida a ubiquinol. Este complejo no trasloca protones a través de la MIM (Palmeira y Moreno, 2012).

Complejo III o citocromo c oxido reductasa. Este complejo es un dímero, está compuesto por 11 subunidades y contiene dos citocromos tipo b, un citocromo tipo c1 y centros Fe-S. Cataliza la transferencia de electrones desde el ubiquinol a

citocromo c. Trasloca cuatro protones de la matriz hacia el espacio intermembranal mediante el ciclo Q. De manera general, este ciclo regenera el ubiquinol mediante el citocromo c1 y b. El citocromo c transfiere los electrones al complejo IV (Wallace, 2013; Nelson y Cox, 2005).

Complejo IV o citocromo c oxidasa. Es una enzima dimérica que transfiere electrones desde el citocromo c al oxígeno molecular. Es la última enzima de la CTE. Este complejo está formado por 13 subunidades, de ellas, tres son críticas para el transporte de electrones. Contiene dos citocromos (a y a_3) y dos centros cobre (Cu_A y Cu_B). El citocromo c cede los electrones al centro Cu_A y éste a su vez lo cede al citocromo a . Desde ahí los electrones pasan al citocromo a_3 y al centro Cu_B . Por cada dos electrones que atraviesan el complejo IV, bombea dos protones al espacio intermembranal (Nelson y Cox, 2005).

ATP sintasa o F_1F_0 o (complejo V). Cataliza la formación de ATP a partir de ADP y P_i , acompañada de flujo de protones del espacio intermembranal a la matriz mitocondrial. Está constituido por 16 subunidades, las cuales forman dos componentes, F_1 , soluble en la matriz mitocondrial y F_0 , embebido en la MMI. La fracción F_1 es una proteína dirigida hacia la matriz mitocondrial, constituida por nueve subunidades, tres unidades α y tres β . En la fracción F_0 se forma un canal mediante el cual fluyen los protones y promueve la rotación del anillo de la subunidad c los cuales son liberados en la matriz mitocondrial. Este movimiento permite cambios en el componente F_1 responsables de la síntesis de ATP (Nelson y Cox, 2005).

El transporte de electrones impulsa un flujo de protones por los complejos mitocondriales I, III y IV de la matriz mitocondrial hacia el espacio intermembrana generando un gradiente de protones. Este gradiente es empleado para la síntesis de ATP por la ATP sintasa. Este proceso se denomina fosforilación oxidativa. Es decir, la mitocondria acopla dos procesos, la CTE y la fosforilación.

La rigidez de la MMI puede alterar las funciones de los complejos mitocondriales y enzimas, incluyendo aquellas de la CTE (Santos *et al.*, 2007).

La fuga de electrones de la cadena respiratoria mitocondrial es una fuente intracelular de generación de radicales libres. De hecho, es particularmente problemático el resultado de la reducción o el bloqueo de la CTE. Bajo tales condiciones, la producción excesiva de radicales libres y la formación de ERO pueden conducir a lesiones y a la muerte celular (Yang *et al.*, 2014).

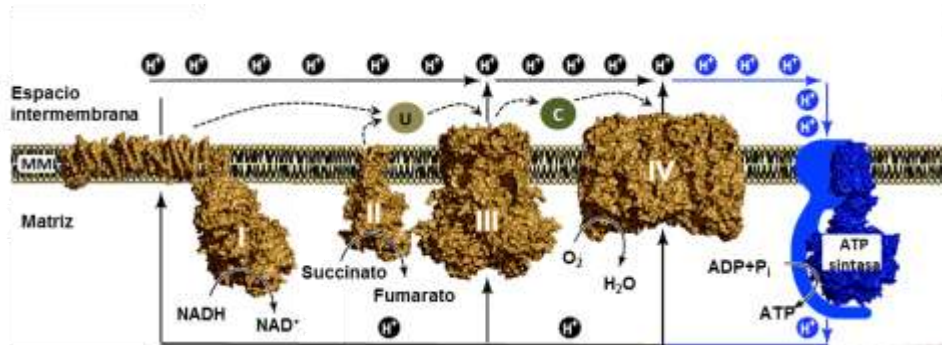


Figura 6. Cadena de transporte de electrones (CTE) acoplada a la síntesis de ATP. El flujo de electrones (representado por las líneas punteadas) entre los complejos mitocondriales y el bombeo de protones desde la matriz hacia el espacio intermembranal, por los complejos I, III y IV, generan un gradiente de protones. Este gradiente es empleado por la ATP sintasa para fosforilar ADP y generar ATP. La CTE está representada por las líneas negras, mientras que la fosforilación oxidativa está representada por las líneas azules. Los complejos I, II, III y IV son: NADH deshidrogenasa, succinato deshidrogenasa, citocromo c oxidoreductasa y citocromo c oxidasa, respectivamente, MMI: membrana mitocondrial interna, U: ubiquinona, C: citocromo c. Tomado de Brand *et al.* (2013).

1.9 Acoplamiento mitocondrial

Para evaluar el acoplamiento entre la CTE y la síntesis de ATP se mide el consumo de oxígeno (Palmeira y Moreno, 2012; Marcinek, 2004). El consumo de oxígeno se determina en medio acuoso en una cámara cerrada con temperatura controlada empleando un electrodo tipo Clark con mitocondrias recién aisladas (Terada, 1990).

La gráfica obtenida del consumo de oxígeno presenta diferentes pendientes, que representan diferentes estados de respiración (Figura 7). El estado 1 (edo. 1) corresponde a las mitocondrias en el medio que consumen oxígeno utilizando los sustratos internos, es un estado basal. El estado 2 (edo. 2), se observa tras adicionar un sustrato (malato/glutamato o succinato) en el medio y se incrementa el consumo de oxígeno. El consumo de oxígeno aumenta durante el estado 3 (edo. 3) después de adicionar ADP en el medio con las mitocondrias aisladas en suspensión. El aumento de la respiración denota el uso del gradiente de H^+ para la síntesis de ATP. Cuando todo el ADP adicionado en el medio se agota al fosforilarse a ATP, el consumo de oxígeno decae. Este estado se le conoce como estado 4 (edo. 4). Existe otro estado que se induce al agregar algún desacoplante, disipando el potencial de membrana e incrementa la velocidad de consumo al máximo (Respiración desacoplada) (Palmeira y Moreno, 2012; Lee-Jung, 2012).

El índice de coeficiente respiratorio (ICR) es un buen indicador de la integridad de la MMI y del acoplamiento entre la oxidación de sustratos y la fosforilación oxidativa, se obtiene al dividir el estado 3 entre el estado 4.

Otro parámetro importante es el cociente ADP/O o P/O. Es una medida de eficiencia de la fosforilación oxidativa, representa la cantidad de oxígeno que se reduce a agua por el complejo IV y que se emplea para la síntesis de ATP (Lee-Jung, 2012; Palmeira y Moreno, 2012). Se requieren de 3-4 H^+ para la síntesis de ATP por la ATP sintasa. Así, la transferencia de electrones desde el complejo I al oxígeno, genera la translocación de 10 H^+ de la matriz mitocondrial al espacio intemembranal. Es decir, desde el complejo I se genera la síntesis de 2.4-3.1 moléculas de ATP y desde el complejo II se generan 1.4-1.9 moléculas de ATP (Palmeira y Moreno, 2012).

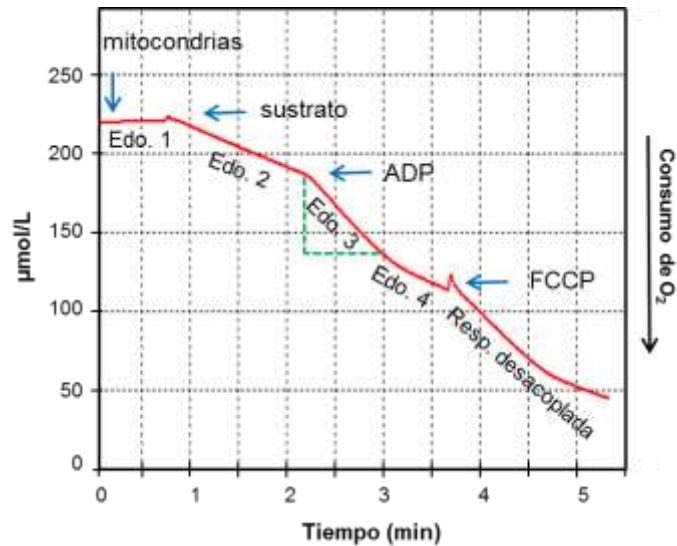


Figura 7. Determinación del consumo de oxígeno. Al agregar las mitocondrias en la cámara, el electrodo tipo Clark determina la concentración de oxígeno en el medio. Se obtienen cuatro estados. El estado 1 (edo. 1) representa el consumo de oxígeno con los sustratos endógenos. El estado 2 (edo. 2) se obtiene tras agregar un sustrato (malato/glutamato o succinato). El estado 3 (edo. 3) se obtiene al agregar ADP y se incrementa el consumo de oxígeno. Al agotarse el ADP, tras ser fosforilado a ATP, el consumo de oxígeno se reduce, a este estado se denomina estado 4 (edo. 4). La respiración desacoplada (Resp. desacoplada) se obtiene al agregar al medio el desacoplante FCCP.

1.10 Disfunción mitocondrial inducida por CP

Como se mencionó anteriormente, la disfunción mitocondrial es un evento temprano en el daño renal inducido por CP (Santos *et al.*, 2007).

Este daño se ha caracterizado por alteraciones en el consumo de oxígeno, incremento de proteínas carboniladas, reducción del contenido de GSH, incremento de la producción de ERO, baja actividad de la enzima aconitasa, disminución de los niveles de ATP, disminución del potencial de membrana, atenuación del ICR y P/O y reducción de la acumulación de calcio (Ca^{2+}) en las mitocondrias renales de ratas tratadas con CP (Rodrigues *et al.*, 2011; Santos *et al.*, 2007; Gordon y Gattone, 1986).

1.11 Antecedentes de CP

Se ha demostrado que el CP se acumula en los túbulos proximales del riñón. En consecuencia, se induce estrés oxidante que ocasiona daño celular y finalmente disfunción renal. Estudios *in vitro* e *in vivo* demuestran las diversas ERO involucradas como $O_2^{\cdot-}$, H_2O_2 y $ONOO^-$ están involucradas en la nefrotoxicidad por CP (Pabla y Dong, 2008; Guerrero-Beltrán *et al.*, 2010).

El incremento de ERO por CP (Paradies *et al.*, 2010; Cullen *et al.*, 2007), esta asociado a la disminución de la actividad de enzimas antioxidantes y del contenido de GSH (Pillai *et al.*, 2011; Yao *et al.*, 2007; Ozen *et al.*, 2004), de esta manera inactiva el sistema antioxidante celular (Sugiyama *et al.*, 2011). Este desbalance de antioxidantes y ERO ocasionan daño oxidante y como resultado daño al ADN, proteínas, lípidos y disfunción mitocondrial están implicados en la patogénesis renal aguda (Firdous y Kuttan, 2012; Konigsberg, 2008). Así, se ha propuesto que el empleo de productos de origen alimenticio con capacidad antioxidante podría ser una alternativa para reducir los efectos no deseados del CP (Tabla 2).

Tabla 2. Compuestos de origen alimenticio que han logrado reducir el daño oxidante inducido por CP en ratones.

Antioxidante	Modelo de daño renal	Referencia
Té negro	Ratón macho ICR (20 mg/kg i.p.)	Hung <i>et al.</i> (2007)
Vitamina E y C	Ratón macho Swiss (12 mg/kg i.p.)	Ajith <i>et al.</i> (2009)
Ácido tánico	Ratón macho Swiss (7 mg/kg i.p.)	Ahmad y Sultana (2011)
(E)- β -cariofileno	Ratón macho C57Bl/6J (25 mg/kg i.p.)	Horváth <i>et al.</i> (2012)
Meso-zeaxantina	Ratón macho Swiss (16 mg/kg)	Firdous y Kuttan <i>et al.</i> (2012)

1.12 Antecedentes de FC

Actualmente, existen varios estudios de la FC que evalúan su efecto protector contra el estrés oxidante inducido por fármacos.

Romay *et al.* (1998a) demostraron que la FC tiene propiedades anti-inflamatorias. Utilizaron un modelo de inflamación aguda con carragenina (subplantar) y ácido araquidónico (aplicación tópica en orejas) en rata y ratón, respectivamente. La administración i.g. a la dosis de 50, 100 y 200 mg/kg fue 1 hora antes de inducir la inflamación. Una hora posterior a la inducción, sacrificaron a los animales, midiendo dicho proceso por la diferencia de peso de las orejas. En el caso del modelo con carragenina, se determinó el aumento del volumen de las extremidades con la ayuda de un vernier después de cinco horas. Se concluyó que en ambos modelos animales los mejores resultados se obtuvieron con las dosis de 100 y 200 mg/kg.

Lim *et al.* (2012), encontraron que el pre-tratamiento de FC a dosis de 50 mg/kg i.p. una hora antes de CP 12 mg/kg i.p. protege contra el daño nefrotóxico en ratones C57BL6. Después de 72 h los ratones se sacrificaron y se determinó que disminuye significativamente la muerte por apoptosis.

Los resultados de este estudio y de otros afines al tema se resumen en la tabla 3.

Tabla 3. Modelos *in vivo* e *in vitro* de las propiedades de la FC.

Propiedad	Dosis Fc	Referencia
Anti-inflamatoria	Dosis oral de 50, 100, 200 mg/kg 1 hora antes	Romay <i>et al.</i> (1998a)
Hepatoprotector	Dosis 100, 200 y 400 mg/kg oral por 7 días	Ou <i>et al.</i> (2010)
Renoprotector	Dosis 50 mg/kg i.p. 1 hora antes de CP 12 mg/kg i.p.	Lim <i>et al.</i> (2012)
Inhibidor selectivo COX-2	5-20 μ M por 48 horas en macrófagos	Reddy <i>et al.</i> (2003)
Reduce cardiotoxicidad	Pre-tratamiento con 10 μ M por 1 h y co-incubación con Dox 24 h horas	Khan <i>et al.</i> (2006)

Con base en los antecedentes antes citados, planteamos la siguiente justificación.

2 JUSTIFICACIÓN

El CP es un fármaco eficaz en el tratamiento de tumores sólidos. Sin embargo, su efecto secundario más importante es la nefrotoxicidad relacionada con el estrés oxidante. De esta manera y considerando que la FC es un nutraceutico con capacidad antioxidante, se propone que este compuesto puede reducir los efectos nefrotóxicos del cisplatino. Nuestro estudio contribuirá a la búsqueda de compuestos de origen natural que puedan emplearse para prevenir el daño renal inducido por CP.

3 HIPÓTESIS

Si la FC atenúa el estrés oxidante y las alteraciones mitocondriales, entonces su administración reducirá la nefrotoxicidad inducida por CP en ratones CD-1.

4 OBJETIVO GENERAL

Determinar si el nutraceutico FC protege contra el daño renal inducido por cisplatino en ratones CD-1 e investigar el mecanismo de acción mediante la determinación de enzimas antioxidantes, el daño oxidante y la función mitocondrial.

5 OBJETIVOS ESPECÍFICOS

- Establecer la dosis de CP que induzca daño renal en ratones CD-1.
- Evaluar si la FC presenta un efecto protector sobre la nefrotoxicidad inducida por CP en un modelo *in vivo*.
- Determinar la actividad de las enzimas antioxidantes y marcadores de estrés oxidante en el homogenizado renal.
- Evaluar la función mitocondrial mediante el consumo de oxígeno, la síntesis de ATP, daño oxidante y enzimas antioxidantes en mitocondrias renales.

6 METODOLOGÍA

6.1 Reactivos

La FC fue donada por Parry Nutraceuticals (EID Parry (I) Ltd., India). Los siguientes reactivos se adquirieron de Sigma-Aldrich (St. Louis, MO, EUA): CP (Cat. no. P4394, Lot# MKBG8465V), 2,2'-azobis(2-amidinopropano) dihidrocloruro (AAPH), xantina, xantina oxidasa, nitro azul de tetrazolio (NBT), fluoresceína, DL-penicilamina, ácido dietil-triamino-penta-acético (DTPA), 2,2-difenil-1-picrilhidrazina (DPPH), ácido tereftálico (DTPA), ácido ascórbico, amplex red, peroxidasa de rábano, piruvato, dimetil tiourea (DMTU), nicotinamida adenina dinucleótido reducido (NADPH), ácido lipoico, glutatión reducido (GSH), 1-cloro-2,4,-dinitrobenzoceno (CDNB), p-nitrofenil-N-acetil-β-D-glucosaminida, tetrametoxipropano, monoclorobimano, 1-metil-2-fenil indol, ácido metasulfónico, dimetilsulfóxido (DMSO), 1,3-difenil-iso-benzofurano (DPBF), ácido nordihidroguaiarético (NDGA), glutatión oxidado (GSSG), ácido p-aminobenzoico, dodecil sulfato de sodio (SDS), poliacrilamida, D-manitol, succinato de sodio dibásico, ácido 4-(2-hidroxietil) piperacina-1-etanosulfónico (HEPES), adenosina-5'-difosfato de sodio (ADP), carbonilcianuro-p-trifluorometoxi-hidrazona (FCCP), percoll, sacarosa, nicotinamida adenina dinucleótido (NADH). Otro frasco de CP (Cat. no. 479306-1G; Lote: MKBH5984V) fue adquirido de Aldrich (St. Louis, MO, EUA.) La 1,2,3 dihidrorodamina (DHR-123) se adquirió de Cayman Chemical Co. (Ann Arbor, MI, EUA). El trolox se adquirió de EMD Millipore (Billerica, MA, EUA). La sal sódica del ácido etilendiaminotetraacético (Na₂-EDTA), el hipoclorito de sodio (NaClO) y el H₂O₂ se compraron en JT Baker (Xalostoc, Edo. México, México). Los kits para la determinación de creatinina plasmática y BUN se adquirieron de Spinreact (Girona, España). El resto de los reactivos fueron grado analítico y se adquirieron comercialmente.

6.2 Determinación de la pureza de la ficocianina

Para determinar la pureza de FC de la muestra, se realizaron lecturas en un espectrofotómetro a longitudes de onda de 280 y 620 y se calcularon con la siguiente ecuación (Patel *et al.*, 2005):

$$\text{Pureza a ficocianina} = \frac{\text{Absorbancia}_{620}}{\text{Absorbancia}_{280}}$$

6.3 Electroforesis en geles de poliacrilamida

Se realizó una electroforesis en gel de poliacrilamida con SDS al 15%, utilizando azul de Coomassie 0,1% como colorante para identificar las dos sub-unidades proteicas (α y β). Se emplearon marcadores de peso molecular de Precisión Plus (Bio-Rad).

6.4 Actividad atrapadora *in vitro*

Las muestras de FC y de los compuestos estándares que se emplearon fueron medidas en un lector de microplacas (Biotek Instruments, Winooski, VT, EUA).

6.4.1 Actividad atrapadora de H₂O₂

Se evaluó usando el reactivo Amplex red. Se incubaron 25 μ L de FC con 25 μ L de 10 μ M de H₂O₂ por 20 min, posteriormente se adicionaron 50 μ L de la mezcla de reacción que contenía 0.1 mM de Amplex red y 0.2 U/mL HRP. La mezcla se incubó (sin luz) otros 20 min (Floriano-Sánchez *et al.*, 2006). La concentración de H₂O₂ de la muestra fue directamente proporcional a la fluorescencia obtenida en el ensayo.

6.4.2 Actividad atrapadora de OH[•]

Esta prueba se basó en que el compuesto no fluorescente tereftalato que al reaccionar con el radical hidroxilo se produjo un compuesto fluorescente. Se incubó por 30 minutos a temperatura ambiente con ácido ascórbico 1 mM, FeCl₃ 1 mM, EDTA 1 mM, H₂O₂ 10 mM y tereftalato 1.4 mM (Floriano-Sánchez *et al.*,

2006). El producto fluorescente se determinó en el lector de placas Synergy HT a 326 nm de excitación y 432 nm de emisión.

6.4.3 Actividad atrapadora de $O_2^{\cdot-}$

Se empleó el sistema de xantina-xantina oxidasa para generar el radical superóxido, el cual reduce NBT a formazán, que se lee a 560 nm (Floriano-Sánchez *et al.*, 2006).

6.4.4 Actividad atrapadora de ROO^{\cdot}

Se empleó AAPH para generar el radical, el ensayo consistió en adicionar 25 μ L de FC utilizando diferentes concentraciones a 25 μ L de una mezcla con 150 μ L de 30 mM de fluoresceína y 25 μ L de AAPH 38.5 mM (Floriano-Sánchez *et al.*, 2006). La fluorescencia se midió a 485 nm de excitación y 520 nm de emisión por 1.5 h a 37°C.

6.4.5 Actividad atrapadora de $ONOO^-$

Se emplearon concentraciones conocidas de $ONOO^-$ para determinar la actividad antioxidante de la FC contra este anión, para lo cual se midió la oxidación de DHR123 al producto fluorescente rodamina el cual se midió a 500 y 560 nm de excitación y emisión, respectivamente (Floriano-Sánchez *et al.*, 2006).

6.4.6 Actividad atrapadora de 1O_2

A partir de NaOCl y H_2O_2 se generó el 1O_2 el cual produjo una disminución en la fluorescencia de difenil benzofurano (DFBF, excitación 410 nm y emisión 455 nm) debido a que es un atrapador específico de 1O_2 . Se colocaron 25 μ L de la muestra de FC con 25 μ L: de H_2O_2 (100 mM), DFBF (10 μ M), NaOCl (1 mM) y 150 μ L de etanol (Floriano-Sánchez *et al.*, 2006).

6.4.7 Determinación del CI_{50}

La actividad antioxidante de la FC se expresó como CI_{50} , es decir, la concentración de FC requerida para inhibir en un 50% la concentración de la

especie probada en cada sistema específico mediante una regresión lineal en al menos 3 experimentos independientes.

6.5 Modelo experimental *in vivo*

Seguimos los lineamientos de la Norma Oficial Mexicana de cuidado y uso de los animales de laboratorio (NOM-062-ZOO-1999) y la Norma Oficial Mexicana NOM-087-ECOL-SSA1-2002 de Residuos peligrosos biológico-infecciosos- Clasificación y especificaciones de manejo. Además, este protocolo fue aprobado por el comité de ética del Comité Institucional de Uso y Cuidado de los Animales de Laboratorio de la Facultad de Química (FQ/CICUAL/043/12).

Los animales se mantuvieron con ciclos de luz oscuridad de 12 h cada uno a una temperatura de 21°C, con una humedad relativa de 50%, se alimentaron con alimento estándar y agua *ad libitum*.

Se emplearon 54 ratones machos CD-1 (30-35 g). Se dividieron en 6 grupos de 9 ratones cada uno:

Grupo T	Testigo, solución salina inyectada intraperitonealmente (i.p.) como vehículo
Grupo FC30	FC 30 mg/kg i.p. 1 h antes de la solución salina i.p.
Grupo CP	Solución salina i.p. 1 h antes de CP 18 mg/kg i.p.
Grupo FC5+CP	FC 5 mg/kg i.p. 1 h antes de CP 18 mg/kg i.p.
Grupo FC10+CP	FC 10 mg/kg i.p. 1 h antes de CP 18 mg/kg i.p.
Grupo FC30+CP	FC 30 mg/kg i.p. 1 h antes de CP 18 mg/kg i.p.

Al final del tratamiento (72 h después de la dosis de CP) los animales se anestesiaron con pentobarbital (70 mg/kg i.p.), posteriormente se obtuvo la sangre por plexo axilar usando tubos eppendorf con heparina como anticoagulante. Los ratones se dislocaron cervicalmente y se extrajeron los riñones derechos que se

congelaron inmediatamente en nitrógeno líquido hasta su procesamiento. El riñón izquierdo se colocó en formalina al 10% para el estudio histológico.

6.5.1 Determinación del daño renal

Las determinaciones de BUN y creatinina se realizaron con los kits comerciales. La actividad de la enzima N-acetil- β -D-glucosaminidasa (NAG) se determinó a 405 nm usando p-nitrofenil-N-acetil- β -D-glucosamínido como sustrato, los datos se expresaron como U/mg de proteína. Una unidad de NAG se definió como la cantidad de enzima que libera 1 μ mol de p-nitrofenol por min en las condiciones del ensayo.

6.5.2 Análisis histopatológico

Los riñones se deshidrataron en concentraciones ascendentes de etanol, se embebieron en parafina y se seccionaron a un espesor de 4 micras, posteriormente se tiñeron con hematoxilina y eosina (H&E). Las laminillas se observaron en el microscopio de luz (40X) y se determinaron las alteraciones histológicas de, al menos, 30 campos/laminilla elegidos al azar utilizando morfometría automatizada (Leica Qwin, Cambridge, England). El porcentaje de daño se obtuvo teniendo en cuenta los cambios histopatológicos tales como edema, vacuolización, descamación, apoptosis y necrosis. El área de las células dañadas y muertas se determinó mediante el cálculo del porcentaje de daño con respecto al área total.

6.5.3 Marcadores de daño oxidante

Del homogenizado renal se evaluaron MDA, 4-HNE, proteínas oxidadas y el contenido de GSH. El contenido de MDA se determinó mediante una curva estándar con tetrametoxipropano (200 μ L) o del homogenizado renal en una solución de acetonitrilo:metanol (3:1) con 1-metil-2-fenilindol 15.4 mM (650 μ L) en presencia de 150 μ l de HCl concentrado. Se incubaron durante 45 min a 40°C. Después las muestras se centrifugaron a 3,000 g por 5 min. Se midió la densidad óptica del sobrenadante a 586 nm. Los valores se informaron como nanomoles de

MDA/g de proteína. El contenido de 4-HNE se evaluó de manera similar que para MDA, usando el ácido metanosulfónico con Fe (III) en vez de HCl. El contenido de GSH renal se obtuvo mediante la formación de aductos fluorescentes con monoclorobimano 1 mM (33 μ L) en presencia de GST 1 U/L (33 μ L). Se empleó una curva estándar de GSH (33 μ L) en solución de Krebs-Henseleit pH 7.4. Los aductos se midieron en un lector de placas Synergy a 385 y 478 nm de excitación y emisión, respectivamente. Los valores se informaron como μ mol/mg de proteína.

6.5.4 Determinación de enzimas antioxidantes

La actividad de la enzima GPx se evaluó mediante la reducción de NADPH, su desaparición se midió a 340 nm en una reacción acoplada que contenía GSH 1 mM, NADPH 0.2 mM y GR 1 U/mL. Brevemente, a 400 μ L de la mezcla de reacción se adicionaron a 50 μ L del homogenizado renal o amortiguador de fosfatos 50 mM pH 7.4 y 15 μ L de H₂O₂ 2.5 mM. Para conocer la actividad de la enzima GST se mezclaron 20 μ L del homogenizado con una solución compuesta de 910 μ L de amortiguador de fosfatos 50 mM pH 6.5, 20 μ L de GSH 2 mM y 50 μ L de CDNB 1 mM. La actividad de GR se midió con base en la desaparición de NADPH, la lectura se hizo a 340 nm. Se mezclaron 50 μ L de la muestra renal con 950 μ L de la mezcla de reacción (GSSG 1.25 mM, NADPH 0.1 mM, EDTA-Na₂ 0.5 mM en amortiguador de fosfatos 100 mM pH 7.6). La actividad de la enzima CAT se determinó por la desaparición de H₂O₂ 30 mM (725 μ L) en presencia de 25 μ L del homogenizado renal. Se realizaron lecturas a 240 nm cada 15 s durante 3 min. Los datos se expresaron como U/mg de proteína.

6.6 Estudios en mitocondrias

Las metodologías empleadas para el aislamiento de mitocondrias, oximetría, síntesis de ATP, manejo de Ca²⁺, potencial de membrana, daño oxidante y enzimas antioxidantes mitocondriales se encuentran en la sección de Anexos, apartados 12.1 al 12.1.7.

6.7 Análisis estadístico

Los resultados se presentan como la media \pm error estándar (E.E.). Se empleó la prueba estadística ANOVA de una vía seguida de comparaciones por el método de Bonferroni. La prueba t de student se utilizó para comparar los resultados del análisis histológico. Se utilizó el programa estadístico SigmaPlot v 11.0 (Systat Software, San Jose, CA, EUA). Los valores de $P < 0.05$ se consideraron significativos.

7 RESULTADOS

7.1 Pureza de FC “Parry Nutraceuticals” y electroforesis de proteínas en geles de poliacrilamida

El espectro de absorción reveló que la FC de Parry nutraceuticals tiene una pureza de 2.5 ($A_{620\text{nm}}/A_{280\text{nm}}$) que corresponde a un grado reactivo. Se observaron las dos bandas características las sub unidades de FC α y β (Figura 8) en la electroforesis de proteínas.

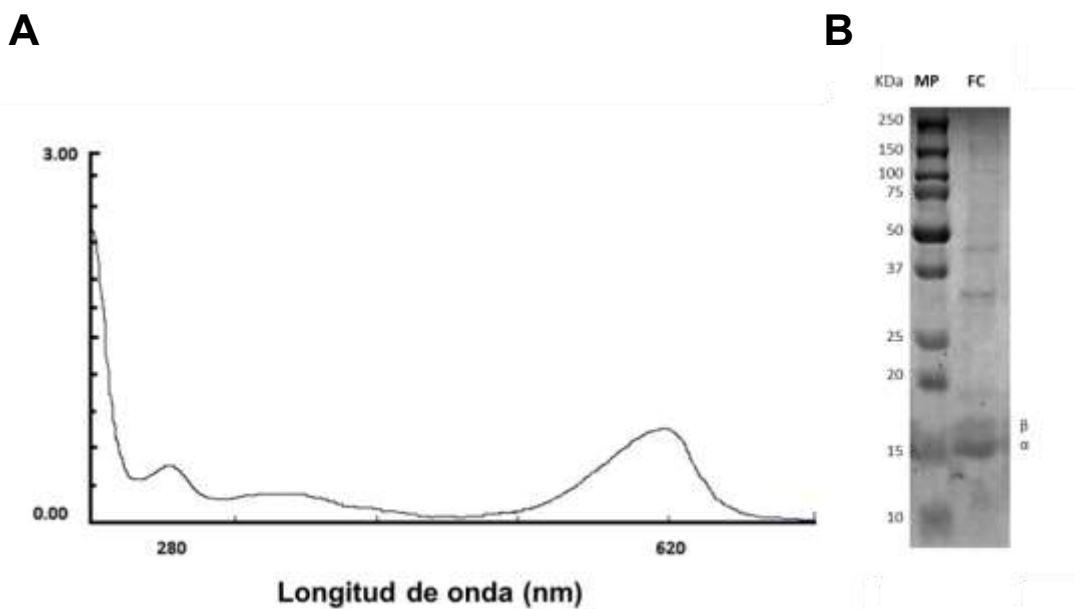


Figura 8. Espectro de absorción en amortiguador de fosfatos 5 mM pH 7.4, en donde su absorción corresponde de la FC empleada a 620 nm para la ficocianilina y 280 nm para las proteínas. **B:** Electroforesis SDS-PAGE con marcadores de peso molecular de precisión (precisión Plus de Bio-Rad en KDa). Se aprecian las bandas α y β características de la FC, con un PM de 11.4 y 14.3 KDa, respectivamente.

7.2 Actividad atrapadora de radicales libres

La muestra de FC se sometió a diferentes ERO y a ONOO^- , con el fin de evaluar la capacidad atrapadora de radicales específicos. Los resultados se presentan en la Tabla 4. Se encontró que la FC atrapó todas las especies reactivas con las que fue retada y de manera destacable, atrapa las especies (O_2^- , H_2O_2 , ONOO^-).

Tabla 4. Actividad antioxidante *in vitro* de la muestra de FC.

ERO	CI_{50} FC ($\mu\text{g/mL}$)	CI_{50} referencia ($\mu\text{g/mL}$)	Compuesto de referencia
ONOO^-	5.00 ± 0.56	1.01 ± 0.26	Penicilamina
ROO^\cdot	20.33 ± 0.56	1.19 ± 0.03	Trolox
OH^\cdot	334.0 ± 9.2	2.6 ± 0.62	DMTU
O_2^-	450 ± 4.72	0.18 ± 0.02	NDGA
$^1\text{O}_2$	$11,670 \pm 0.07$	214.00 ± 85.01	Ácido lipoico
H_2O_2	$21,757.72 \pm 2,302.38$	0.28 ± 0.01	Piruvato

NDGA: ácido nordihidroguaiarético, DMTU: Dimetil urea. Los datos están expresados como media \pm E.E., n=3.

7.3 Modelo *in vivo*

La dosis de CP que indujo el daño renal, se determinó mediante una curva dosis-efecto de creatinina y BUN en plasma de ratones con diferentes dosis i.p. de CP (Figura 9 y Tabla 5). Existió diferencia significativa a partir de la dosis de 15 mg/Kg; por lo que se escogió trabajar con la dosis de 18 mg/Kg.

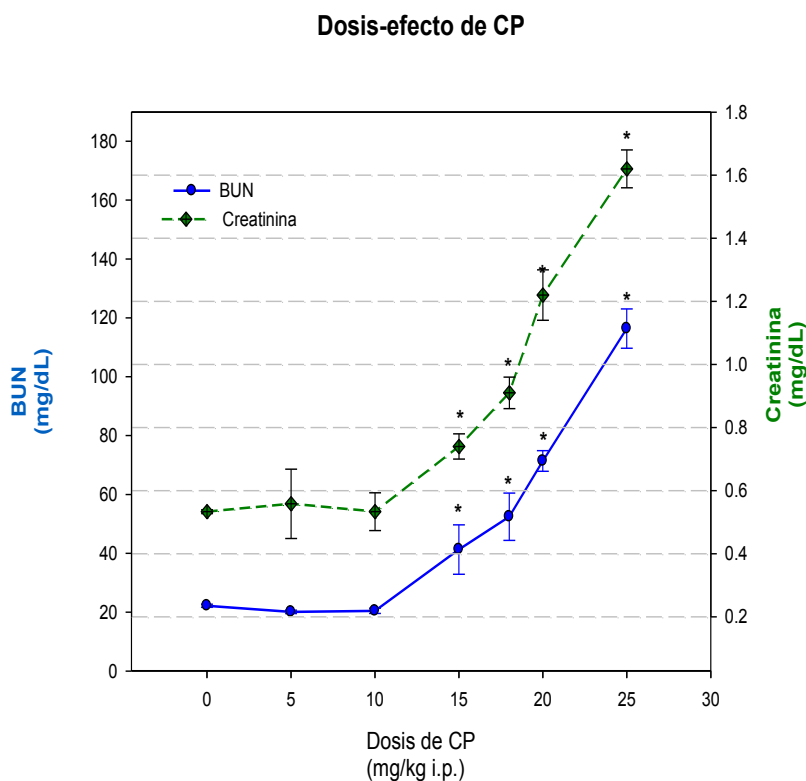


Figura 9. Curva dosis-efecto de CP i.p. en ratones CD-1,* P<0.05 contra testigo de CP.

Tabla 5. Datos de BUN y creatinina plasmático en ratones CD-1 a diferentes dosis de CP i.p.

Dosis CP mg/kg i.p.	BUN (mg/dL)	Creatinina (mg/dL)	N
0	22.20±0.52	0.53±0.08	6
5	20.10±0.58	0.56±0.11	6
10	20.46±0.90	0.53±0.06	6
15	57.06±8.42*	0.76±0.04*	6
18	61.82±7.32*	0.93±0.05*	6
20	72.86±3.42*	1.22±0.08*	6
25	105.60±6.69*	1.63±0.06*	6

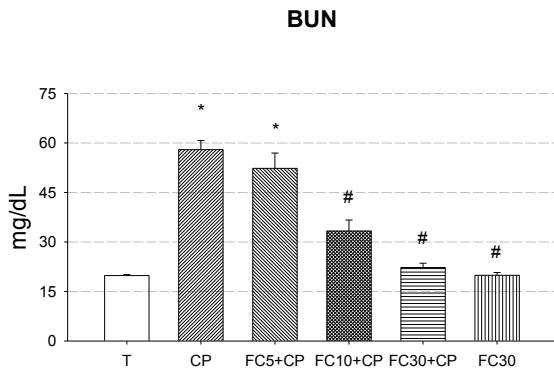
* P<0.05 contra testigo.

7.3.1 Determinación de la función renal

Después de 72 h de haber administrado la dosis de CP (18 mg/kg i.p.) se encontraron alteraciones en los marcadores de la función renal. Se observó un incremento significativo (2 a 3 veces) en los niveles plasmáticos de BUN ($P < 0.05$) y creatinina ($P < 0.001$) en los ratones tratados con CP respecto al testigo. El daño se previno cuando los ratones se trataron con FC de manera dosis-dependiente (Figura 10). En el homogenizado renal del grupo tratado con CP la actividad de NAG disminuyó 34% respecto al control ($P < 0.05$), evidenciando el daño renal. En el grupo de ratones tratado únicamente con FC no se alteró ninguna de las determinaciones antes mencionadas.

7.3.2 Análisis histológico

Los resultados anteriores concuerdan con los observados en las laminillas teñidas con H&E. La microscopía óptica reveló alteraciones estructurales específicamente en los túbulos proximales de los ratones tratados con CP. Las alteraciones se caracterizaron por edema celular, necrosis, apoptosis y la muerte por el desprendimiento de células (Figura 11). El análisis automatizado de morfometría reveló que el $52 \pm 5\%$ de los túbulos proximales presentaron estas anomalías histológicas, y cada túbulo dañado exhibió $58 \pm 8\%$ del área de superficie del epitelio dañado ($p < 0.05$ vs CT). Estas alteraciones se previnieron en el grupo FC30+CP (11%, $p < 0.001$ vs CP). Además no hubo alteraciones en el grupo FC (30 mg/kg).

A**B**

Col 2
Col 7
Col 12
Col 17
Col 22
Col 27

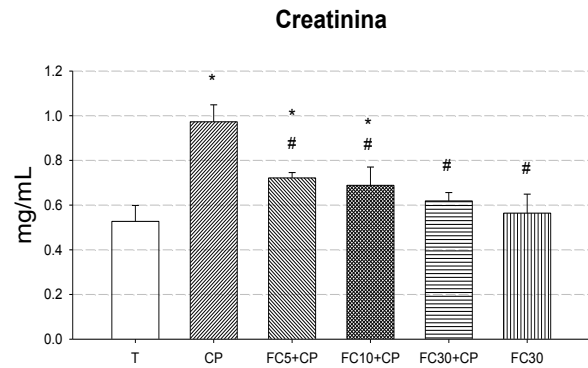
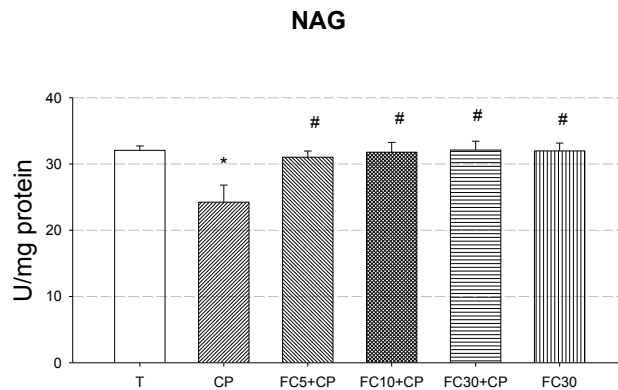
**C**

Figura 10.- Prevención del daño renal inducido por CP en ratones CD-1 tratados con FC. **A:** Nitrógeno de urea en sangre (BUN), **B:** Creatinina plasmática, **C:** N-acetil- β -D-glucosaminidasa (NAG) en homogenizados renales. T: testigo, CP: cisplatino 18 mg/kg i.p, FC 5, 10, 30: ficocianina 5, 10, 30 mg/kg i.p., respectivamente. Los datos están expresados como media \pm E.E., n=5-6. *P<0.05 vs T, #P<0.05 vs CP.

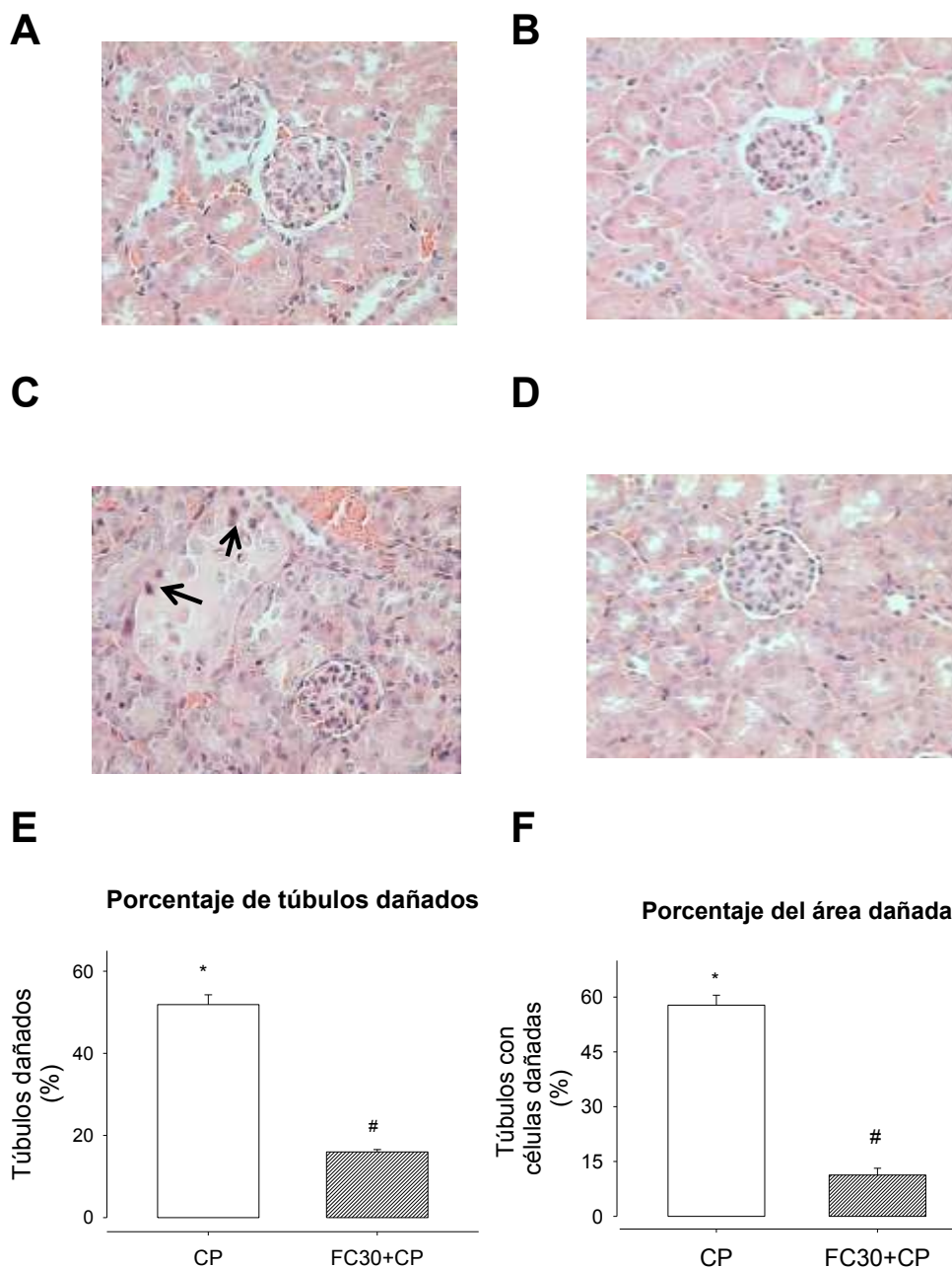


Figura 11. Fotografías representativas del análisis histológico teñido con H&E. **A:** control (CT), **B:** FC30, **C:** CP en donde las flechas indican células muertas, **D:** FC30+CP. T: testigo, CP: cisplatino 18 mg/kg i.p., FC 30: ficocianina 30 mg/kg i.p., respectivamente. Los datos están expresados como media±E.E., n=4. *P<0.05 vs T, #P<0.05 vs CP.

7.3.3 Marcadores de daño oxidante

Los marcadores de daño renal aumentaron en el grupo tratado con CP. El CP indujo una disminución marcada en el contenido de GSH -25% (P<0.001) y un claro aumento en el contenido de 126% en MDA (P<0.001), 358% en 4-HNE

($P < 0.05$) y 42% de proteínas oxidadas ($P < 0.001$, Figura 12) respecto al testigo. Estos efectos se previnieron con FC de forma dosis-dependiente cuando se inyectó una hora antes de CP ($P < 0.05$ vs CP).

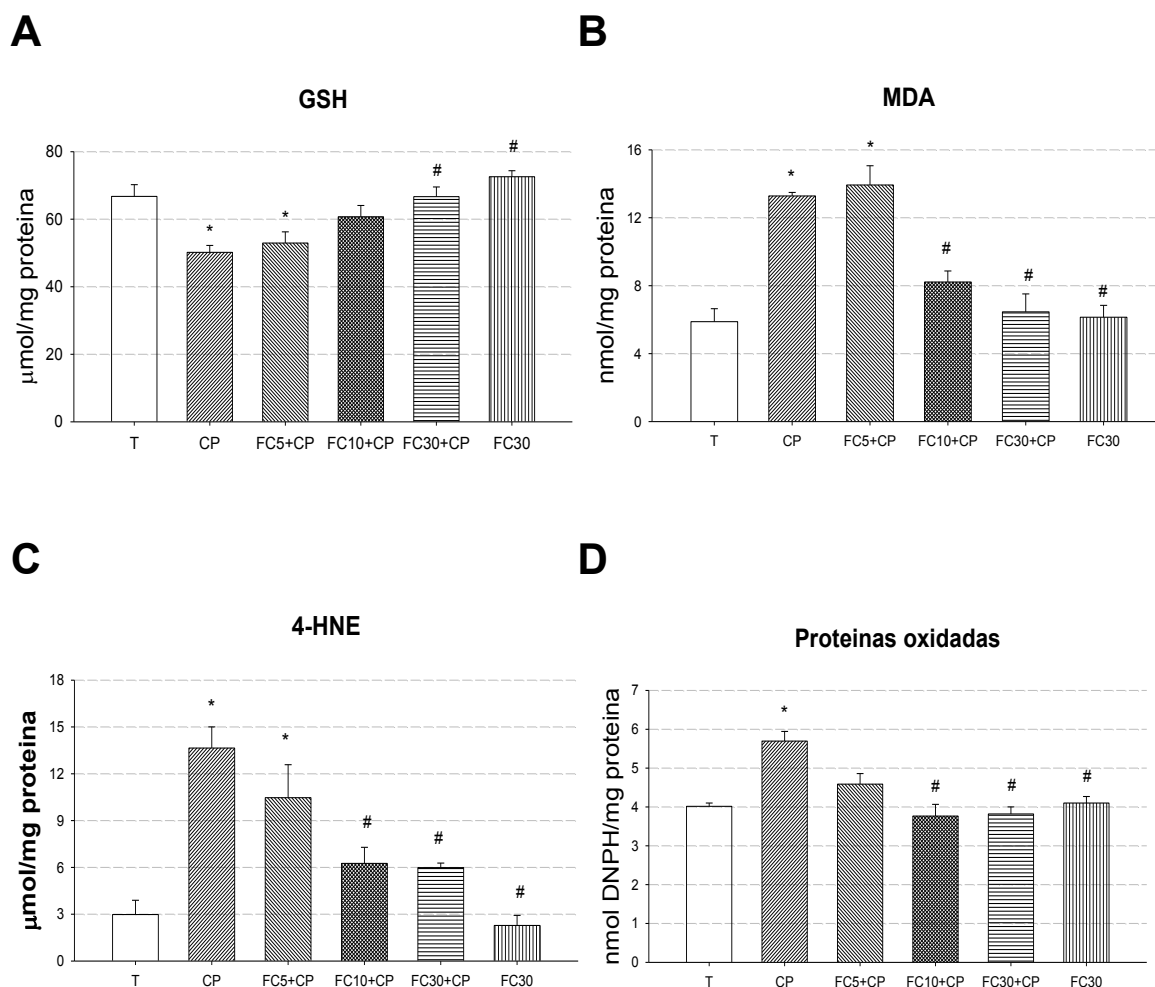


Figura 12.- La FC previene la disminución en el contenido de GSH y el incremento de los marcadores de daño por estrés oxidante en homogenizados renales de ratones CD-1. **A:** Glutación (GSH), **B:** Malondialdehído (MDA), **C:** 4-hidroxi-2-nonenal (4-HNE), **D:** Proteínas oxidadas. T: testigo, CP: cisplatino 18 mg/kg i.p., FC 5, 10, 30: ficocianina 5, 10, 30 mg/kg i.p., respectivamente. Los datos están expresados como media±E.E., n=4-9. * $P < 0.05$ vs T, # $P < 0.05$ vs CP.

7.3.4 Determinación de las enzimas antioxidantes

En el grupo tratado con CP disminuyó significativamente la actividad de enzimas antioxidantes: GPx, GST, GR y CAT en: 40, 23, 40 y 11%, con respecto al grupo

testigo ($P < 0.001$ vs T, Figura 13). Mientras que los grupos pre-tratados con FC 5, 10 y 30 mg/kg i.p. una hora antes de CP prueban que la FC previno la reducción de la actividad con tendencia dosis-dependiente.

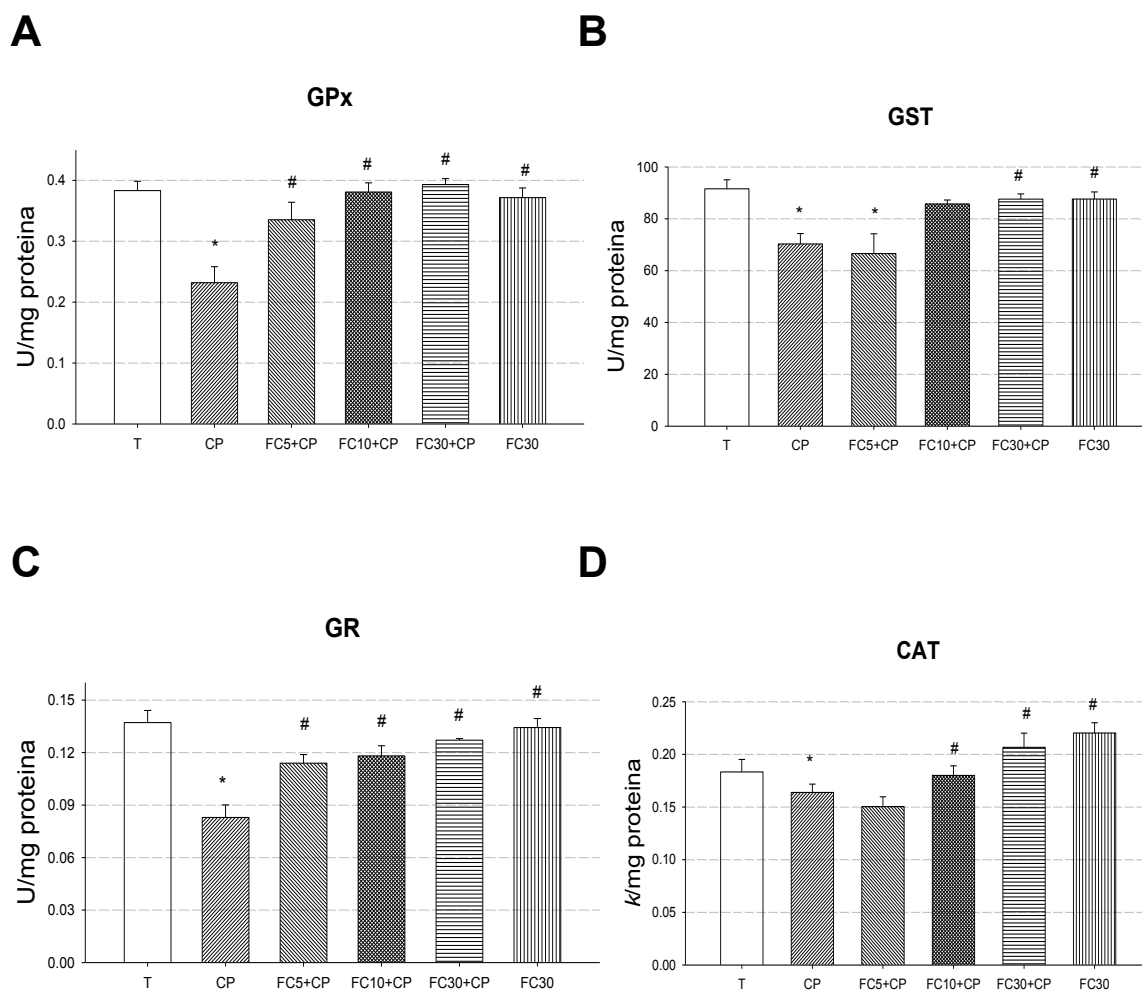


Figura 13. La FC previno la disminución de la actividad antioxidante de homogenizados renales. **A:** Glutación peroxidasa (GPx), **B:** Glutación-S-transferasa (GST), **C:** Glutación reductasa (GR), **D:** Catalasa (CAT). T: testigo, CP: cisplatino 18 mg/kg i.p., FC 5, 10, 30: ficocianina 5, 10, 30 mg/kg i.p., respectivamente. Los datos están expresados como media±E.E., n=4-9. * $P < 0.05$ vs T, # $P < 0.05$ vs CP.

Una vez que se determinó el daño en el homogenizado renal, se realizaron los experimentos en las mitocondrias aisladas de riñón con la dosis de FC más efectiva (30 mg/kg i.p.).

7.4 Estudios en mitocondrias aisladas

Los resultados obtenidos de las mitocondrias renales se encuentran en la sección de Anexos 12.2.1 a 12.2.6. Se demostró que el CP indujo disfunción mitocondrial, redujo la respiración en el estado 3 y el cociente ADP/O con malato/glutamato y con succinato, disminuyó la síntesis de ATP, indujo una disminución en el potencial de membrana y en capacidad de retención de Ca^{2+} . El pretratamiento con FC redujo significativamente estas alteraciones.

El CP indujo daño oxidante mitocondrial el cual se previno con el pre tratamiento de FC en el grupo FC+CP. Ver sección de Anexos (12.2.7).

El CP indujo una reducción significativa en la actividad de las enzimas antioxidantes GPx y CAT la cual se previno con el pre tratamiento de FC en el grupo FC+CP. Ver sección de Anexos (12.2.8).

8 DISCUSIÓN

El CP es un agente quimioterapéutico utilizado a nivel mundial. Sin embargo, su demanda está limitada debido a la frecuencia alta de nefrotoxicidad. Por este motivo, y considerando la importancia que tiene este medicamento en la práctica clínica, nuestro grupo de investigación trabaja en un modelo *in vitro* e *in vivo*, con el objetivo de identificar productos de origen natural que disminuyan los efectos colaterales de CP.

En estudios previos, se demostró que la FC tiene propiedades benéficas para la salud, es decir, tiene efectos anti-inflamatorios, anti-carcinogénicos y antioxidantes (Fernández-Rojas *et al.*, 2014a). Precisamente, esta última propiedad nos fue de gran interés ya que existe suficiente evidencia que sugiere que el mecanismo adverso por el cual el CP induce el daño renal, está asociado al estrés oxidante secundario al incremento de ERO acompañado de una disminución en la actividad de las enzimas antioxidantes. Entonces resulta factible pensar, que el estrés

oxidante generado por CP pudiera ser disminuido si se reestablece el equilibrio entre los agentes oxidantes y antioxidantes.

El daño renal provocado por CP se caracteriza por un aumento de creatinina plasmática y de BUN, así como una reducción de las enzimas antioxidantes GPx, SOD y CAT. Además, el CP induce un daño oxidante caracterizado por el incremento de MDA, 4-HNE, entre otras. Estas alteraciones pueden prevenirse con el empleo de antioxidantes. Para cumplir con este objetivo, trabajamos con ratones CD-1 pero debido a que no se había estandarizado el modelo de daño renal en el laboratorio, se procedió a trabajar con distintas dosis de CP. Luego de una serie de experimentos, observamos un incremento significativo en los niveles de BUN y creatinina con valores al doble del testigo cuando se usó la dosis de 18 mg/kg, por lo que se decidió trabajar bajo estas condiciones.

Lim *et al.* (2012) demostraron que el pre-tratamiento de 1 h con FC (50 mg/Kg i.p.), antes de administrar una dosis única de CP (12 mg/kg i.p.) en ratones C57BL/6, disminuye la apoptosis inducida por este antineoplásico. Teniendo como antecedente este estudio, realizamos experimentos en los que se indujo el daño renal al usar una dosis de CP de 18 mg/Kg i.p. en ratones CD-1 pre-tratados con FC (5, 10 y 30 mg/kg i.p.).

El daño renal se caracterizó por el aumento de creatinina y BUN después de una sola dosis de CP 18 mg/Kg i.p. Dicho daño se confirmó tanto con la determinación de NAG como por estudios histológicos. La enzima NAG es un marcador específico de daño tubular, y por lo tanto, un marcador del daño inducido por CP. Nuestros datos confirman los encontrados por Lim *et al.* (2012), quienes demostraron la protección renal con FC (50 mg/kg i.p.) en ratones tratados con CP (12 mg/kg i.p.). Sin embargo; somos los primeros en demostrar otros mecanismos de protección de FC, la reducción del daño oxidante, su relación con las enzimas antioxidantes y la prevención de la disfunción mitocondrial como se describe a continuación.

En el homogenizado renal, el contenido de MDA, 4-HNE y proteínas oxidadas, como marcadores del daño oxidante de lípidos y proteínas, respectivamente, fue mayor en el grupo tratado con CP. A diferencia de éstos marcadores, se observó una concentración baja del contenido de GSH, principal antioxidante no enzimático hidrosoluble donde el desbalance de éste afecta la homeostasis de ERO y de antioxidantes endógenos del organismo, por lo tanto su baja concentración refleja daño oxidante. Se encontró que un pre-tratamiento de FC (10 y 30 mg/kg i.p.) disminuye significativamente la presencia de los marcadores de daño oxidante en el homogenizado renal.

El marcado daño oxidante y la reducción del contenido de GSH en el homogenizado renal del grupo tratado con CP, se asoció a la disminución de la actividad de las enzimas antioxidantes GPx, GR, GST y CAT. En parte, esto se explica por el aumento de ERO y/o la reducción del GSH, pues este último es cofactor de GPx y GST. Sin embargo la FC evita significativamente y de forma dosis-dependiente la baja actividad de ellas.

El daño por CP incrementa diversas ERO, tales como $O_2^{\cdot-}$, H_2O_2 , $\cdot OH$ y $ONOO^{\cdot-}$, entre otros (Yao *et al.*, 2007; Ozen *et al.*, 2004). En el estudio de actividad antioxidante *in vitro* de la FC, se demostró que esta proteína es capaz de atrapar estas ERO, lo cual puede explicar la reducción del daño oxidante y le prevención de la pérdida en la actividad de las enzimas antioxidantes antes mencionadas.

Después de demostrar que la FC previene la nefrotoxicidad inducida por CP al reducir el estrés oxidante, nos enfocamos a determinar si la prevención de la disfunción mitocondrial era otro de los mecanismos involucrados en su protección, estos datos, así como su metodología realizada se encuentra en la sección de Anexos.

Para este momento, el CP empleado en los ensayos del homogenizado renal se terminó. Por lo tanto, hicimos uso de un nuevo frasco de CP pero de diferente

marca. Se buscó la dosis que reflejara el daño renal observado en el estudio previo (homogenizado renal), con base en los niveles de creatinina y BUN. La nueva dosis de CP correspondió a 22 mg/kg i.p. Una vez que se comprobó que la FC protegía del daño renal inducido por CP, se comenzaron a realizar los experimentos en las mitocondrias.

Se empleó microscopía electrónica con el objetivo de evaluar nuestro método de aislamiento mitocondrial. El resultado derivado de esta evaluación reveló una pureza alta de las mitocondria aisladas.

Los datos de oximetría revelan que el tratamiento con CP induce alteraciones en los complejos mitocondriales y causan la reducción del consumo de oxígeno del estado 3 con malato:glutamato como con succinato.

Se podría especular que el tratamiento con CP modifica la actividad de los complejos I-IV. El consumo de oxígeno del estado 4 se incrementó con ambos sustratos; es decir, las mitocondrias están desacopladas pues fueron incapaces de incrementar la respiración en presencia de ADP exógeno (estado 3). En consecuencia, el ICR disminuyó. Probablemente, la FC atenuó las alteraciones en la actividad de los complejos mitocondriales inducidos por CP, quizá lo suficiente para reducir las variaciones en los estado 3 y 4 y el ICR con succinato.

La respiración desacoplada inducida con FCCP se reduce significativamente en el grupo de CP, ya que probablemente, la actividad de los complejos mitocondriales (I-IV) están afectados. En el grupo pre-tratado con FC se re-establece la respiración desacoplada (con succinato como sustrato).

En el grupo tratado con CP, el rendimiento de la síntesis de ATP por oxígeno consumido (ADP/O) se reduce, dato que correlaciona perfectamente con la disminución de la síntesis de ATP inducida por CP. Estos cambios se previnieron por la dosis única de 30 mg/kg i.p de FC.

El calcio y las ERO, son inductores de la apertura del poro de transición de la permeabilidad (PTPm). Así, el ensayo de manejo de calcio y el de potencial de membrana mitocondrial, indica que las mitocondrias del grupo CP no tienen la capacidad de almacenar Ca^{2+} de la misma manera que las del grupo testigo, como consecuencia del ingreso del catión el potencial de membrana se reduce provocando la apertura del PTPm y la subsecuente liberación de Ca^{2+} , como consecuencia la disipación del potencial de la membrana mitocondrial (Isenberg y Klauning, 2000). En experimentos que emplearon a la CsA como un inhibidor de la apertura del PTPm, la mitocondria conservó la habilidad de retención de calcio y el potencial de membrana mitocondrial (Isenberg y Klauning, 2000). Estos datos evidencian que las mitocondrias del grupo CP inducen la apertura del PTPm. Estos cambios se previnieron por el tratamiento con FC.

Se ha reconocido la disfunción mitocondrial como un mediador en diferentes patologías, como el envejecimiento celular debido al aumento de ERO que origina daños al ADN, lípidos y proteínas (Paradies *et al.*, 2010; Brand *et al.*, 2013). Aproximadamente el 2% del oxígeno en la mitocondria se reduce a superóxido, principalmente en los complejos I y III, la mayor parte es dismutado de H_2O_2 por SOD mitocondrial (Inoue *et al.*, 2003; Klimova y Chandel, 2008). El H_2O_2 puede atravesar a través de la membrana mitocondrial y más tarde puede dar lugar a la producción de radicales hidroxilo altamente reactivo y al daño oxidante (Ott *et al.*, 2007).

El daño oxidante mitocondrial inducido por CP se caracterizó por una reducción significativa del contenido de GSH y por la reducción en la actividad de aconitasa. En el grupo FC+CP, la FC evitó la reducción de las enzimas antioxidantes CAT y GPx, las cuales degradan al H_2O_2 .

El radical $\text{O}_2^{\cdot-}$ es la ERO responsable del mayor daño oxidante en las mitocondrias, ya que todo el $\text{O}_2^{\cdot-}$ se reduce a H_2O_2 . Ésta última ERO, difunde libremente por la membrana mitocondrial induciendo daño a proteínas y lípidos del

riñón. A la vez, la reducción de GSH disminuye la actividad de GPx que lo emplea como cofactor.

Gupta *et al.* (2011) describieron que la FC (70 mg/kg i.p.) mitiga la despolarización de la membrana mitocondrial y atenúa la apoptosis en timocitos de ratas tratadas con tributil estaño (Gupta *et al.*, 2011). También, se ha demostrado que la FC (200 mg/kg por vía oral) previene la permeabilización de la membrana mitocondrial (Saini *et al.*, 2012). Recientemente, Lim *et al.* (2012) describieron el efecto inhibitorio de FC (50 mg/kg, i.p.) en los eventos apoptóticos inducidos por CP (12 mg/kg, i.p.) en ratones.

Coinsiderando nuestros resultados y los antes descritos, el siguiente paso en nuestra línea de investigación sería determinar la actividad de los complejos mitocondriales y conocer si la FC no interfiere con la acción antineoplásica del CP. Por otro lado, es necesario evaluar si los resultados obtenidos en el presente estudio serían reproducibles en un modelo de cáncer murino y posteriormente en humanos con cáncer, con el fin de prevenir la nefrotoxicidad inducida por CP y quizá otros efectos secundarios.

9 RESUMEN DE RESULTADOS

- La FC utilizada presenta actividad antioxidante contra las ERO involucradas en el daño renal inducido por CP.
- La FC protege de manera dosis-dependiente contra el daño renal inducido por CP en el homogenizado renal en ratones CD-1. Esta protección se asoció con la reducción del daño oxidante y a la disminución de las enzimas antioxidantes (GPx, GST, GR y CAT).
- La FC previene el daño en los siguientes parámetros mitocondriales tanto con malato:glutamato como con succinato, evitó la reducción en: el ICR, la síntesis de ATP, el potencial de membrana y del manejo de calcio, la actividad de las enzimas antioxidantes GPx y CAT, así como en la actividad de aconitasa y del contenido de GSH, marcadores de estrés oxidante.

10 CONCLUSIÓN

La ficocianina previene el daño renal inducido por cisplatino reduciendo el daño oxidante, previniendo la reducción de la actividad de las enzimas antioxidantes, así como la disfunción mitocondrial inducida por cisplatino.

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12 ANEXO

12.1 Aislamiento de mitocondrias renales

En este punto del trabajo se terminó el CP usado en experimentos anteriores (Sigma Cat. no. P4394 lote: MKBG8465V) y se comenzó a emplear un nuevo frasco de CP (Aldrich Cat. no. 479306 lote: MKBH5984V). La dosis del nuevo frasco que indujo un daño similar al CP anterior, fue la dosis de 22 mg/kg i.p. (Figura 1A). Así, los experimentos se realizaron con esta nueva dosis.

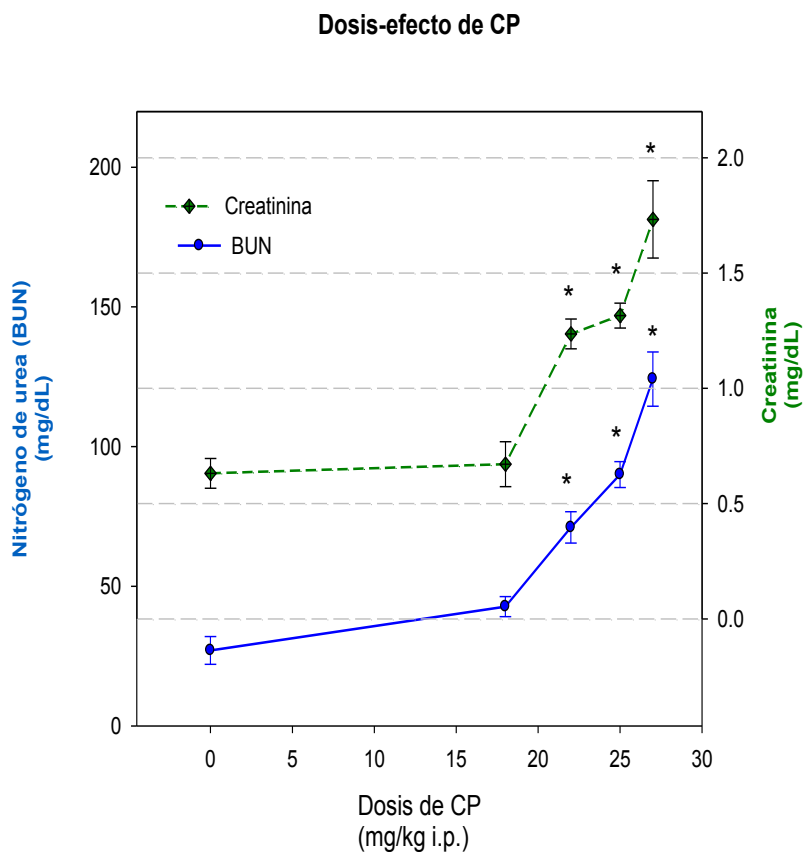


Figura 1A. Dosis efecto del nuevo cisplatino que se empleó en las determinaciones en las mitocondrias aisladas de riñón en ratones CD-1. *P<0.05 vs 0 mg/kg i.p. de CP.

El aislamiento de las mitocondrias se realizó en los grupos: T, FC (30 mg/kg i.p.), CP y FC+CP. Para el fraccionamiento celular, los riñones se colocaron en un homogenizador tipo Potter en amortiguador de aislamiento 1, pH 7.4 (manitol 225 mM, sacarosa 75 mM, HEPES 11 mM, Na₂EDTA 1 mM, BSA 0.5%) en una

relación 10:1 (m:v). Los núcleos y células no homogenizadas se separaron tras centrifugar a 1,100 g por 5 minutos a 4°C. Para la obtención del botón de mitocondrias, el sobrenadante se centrifugó nuevamente en un gradiente de percoll (15-56 %) a 18,500 g por 10 min a 4°C. Con el fin de eliminar el percoll, se realizó un lavado del pellet con 1 mL del amortiguador de aislamiento 2, pH 7.4 (manitol 225 mM, sacarosa 75 mM, HEPES 11 mM, EDTA 1 mM) y se centrifugó a 12,500 g por 10 min. Finalmente el pellet se re-suspendió en 50 μ L del amortiguador de aislamiento 2 (Figura 2A).

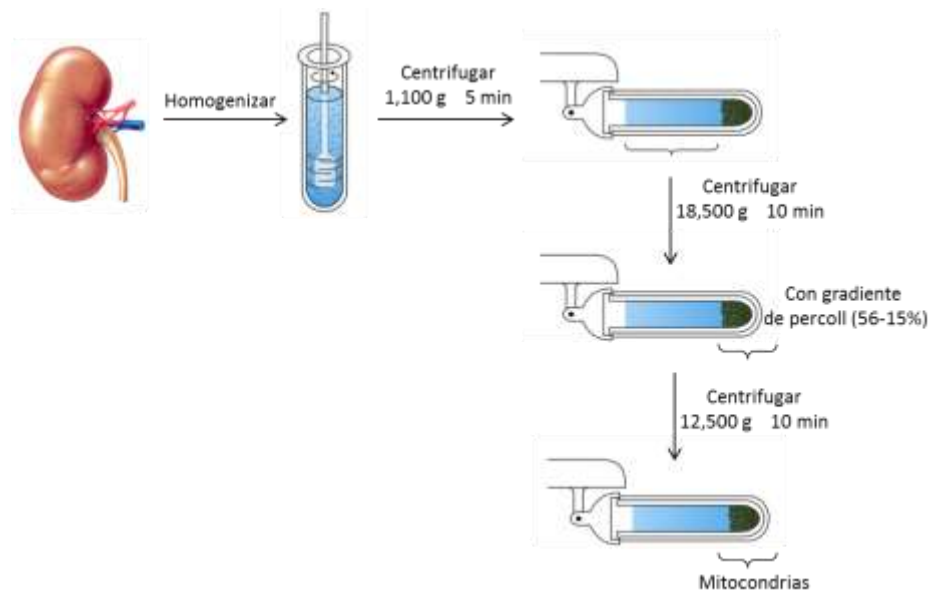


Figura 2A. Diagrama representativo del aislamiento de mitocondrias renales con gradiente de percoll. Basado en ELTE Faculty of Natural Sciences, Institute of Biology Copyright © 2013.

12.1.1 Microscopía electrónica de mitocondrias aisladas

Las mitocondrias aisladas del grupo testigo se obtuvieron mediante el método descrito en la sección 7.7 se fijaron en una solución de glutaraldehído al 10% disuelto en tampón de cacodilato pH 7.2 durante 4 h a 4°C, se fijaron posteriormente en 2% (v/v) de tetróxido de osmio, se deshidrataron en alcohol etílico y se embebieron en resina epoxi. Se obtuvieron secciones ultrafinas con un ultramicrotomo Sorval MT-6000, contrastados con acetato de uranilo y citrato de plomo y se examinaron con un microscopio electrónico FEI-Tecnaí G2.

12.1.2 Oximetría

El consumo de oxígeno se determinó con un electrodo tipo Clark en una cámara de 100 μL a 30°C. Se empleó un medio de respiración que contenía: manitol 220 mM, sacarosa 70 mM, KH_2PO_4 10 mM, MgCl_2 5 mM, HEPES 5 mM, EDTA 1 mM y BSA 0.2%. Como sustrato de respiración se utilizó tanto succinato como malato/glutamato. La determinación se inició al colocar las mitocondrias recién aisladas (10-15 μg de proteína, estado 1), posteriormente se adicionó el sustrato malato/glutamato 10 mM/0.5 mM o succinato 10.5 mM, respectivamente (estado 2) y AD 115 μM (estado 3). Al agotarse el ADP, la respiración llegó al estado 4. En ese momento se adicionó FCCP 0.2 mM para obtener la respiración desacoplada. Todos los datos se ajustaron a la cantidad de proteína adicionada. El ICR se calculó como el cociente del estado 3/estado 4 y la relación ADP/O se obtuvo tras interpolar la cantidad de ADP adicionada por μmol de oxígeno consumido durante el estado 3 (Figura 7).

12.1.3 Síntesis de ATP

Se determinó en mitocondrias recién aisladas mediante reacciones acopladas que se basan en la producción de NADPH a partir del NADP^+ por la enzima glucosa-6-fosfato deshidrogenasa (G6PDH) con glucosa-6-fosfato como sustrato. El medio de reacción empleado contenía: hexocinasa 9 U/mL, glucosa 2.6 mM, G6PDH 2 U/mL, NADP^+ 1.8 μM y succinato 17 mM como sustrato para 15 μg de proteína mitocondrial. La cinética se inició con la adición de ADP 0.24 nM y la absorbancia fue medida a 340 nm por 5 min. El coeficiente de extinción de NADPH usado fue $6,22 \times 10^3 \text{ mM}^{-1}\text{cm}^{-1}$. Finalmente se adicionó oligomicina A (10 μM) para la determinación de la síntesis basal, ésta se resta a la actividad obtenida sin oligomicina.

12.1.4 Retención de calcio

El manejo de Ca^{2+} se evaluó con un espectrofotómetro de doble haz y se graficó el delta de absorbancia entre 675 y 685 nm. Se empleó arsenazo III 28 μM en una mezcla de reacción que contiene: KPi, succinato 10 mM, ADP 0.2 mM y rotenona

1.8 µg/mL. El ensayo inició con la adición de 2 mg de proteína mitocondria (4 ratones) y posteriormente se adicionó CaCl₂ 100 µM. Como control interno del ensayo, se colocó ciclosporina 1 µM al grupo tratado con CP (Martínez-Abundis *et al.*, 2010).

12.1.5 Potencial de membrana

La disipación del potencial de membrana se evaluó con el colorante catiónico safranina O con un espectrofotómetro de doble haz y se graficó la diferencia de absorbancias de 525 y 575 nm. Se realizó de la misma manera que el ensayo de manejo de Ca²⁺, previamente descrito, la diferencia fue el uso de safranina 10 µM (García *et al.*, 2000).

12.1.6 Daño oxidante mitocondrial

Para conocer el daño oxidante en los grupos, se determinó la actividad de la enzima aconitasa y el contenido del GSH en las mitocondrias renales.

12.1.6.1 Aconitasa

La actividad de aconitasa se midió espectrofotométricamente como la formación de cis-aconitato a partir de citrato de sodio 1 mM, cisteína 1 mM, MnCl₂ 0.5 mM, siguiendo la absorbancia a 240 nm empleando un coeficiente de extinción molar de 3.6 mM⁻¹cm⁻¹ (García *et al.*, 2000).

12.1.6.2 Contenido de GSH

El contenido de GSH se determinó del mismo modo que el homogenizado renal, (apartado 7.6.2) con 50 µg de proteína mitocondrial.

12.1.7 Actividad antioxidante mitocondrial

La actividad de las enzimas antioxidantes se determinó del mismo modo que el homogenizado renal empleando 50 µg de proteína mitocondrial (apartado 6.6.3.). Para la determinación de la actividad de la enzima SOD se emplearon 125 µg de proteína mitocondrial en una mezcla de reacción que contenía xantina 0.3 mM, EDTA 0.6 mM, NBT 150 µM, Na₂CO₃ 400 mM y ASB 1%.

12.2 RESULTADOS ANEXOS

12.2.1 Marcadores de daño renal

Después de 72 h de la dosis de CP, se encontró un incremento significativo (2 a 3 veces) en los niveles plasmáticos de creatinina y BUN. El pre-tratamiento con FC (30 mg/Kg i.p. 1 h antes del CP), previno las alteraciones en los marcadores de la función renal (Figura 3A).

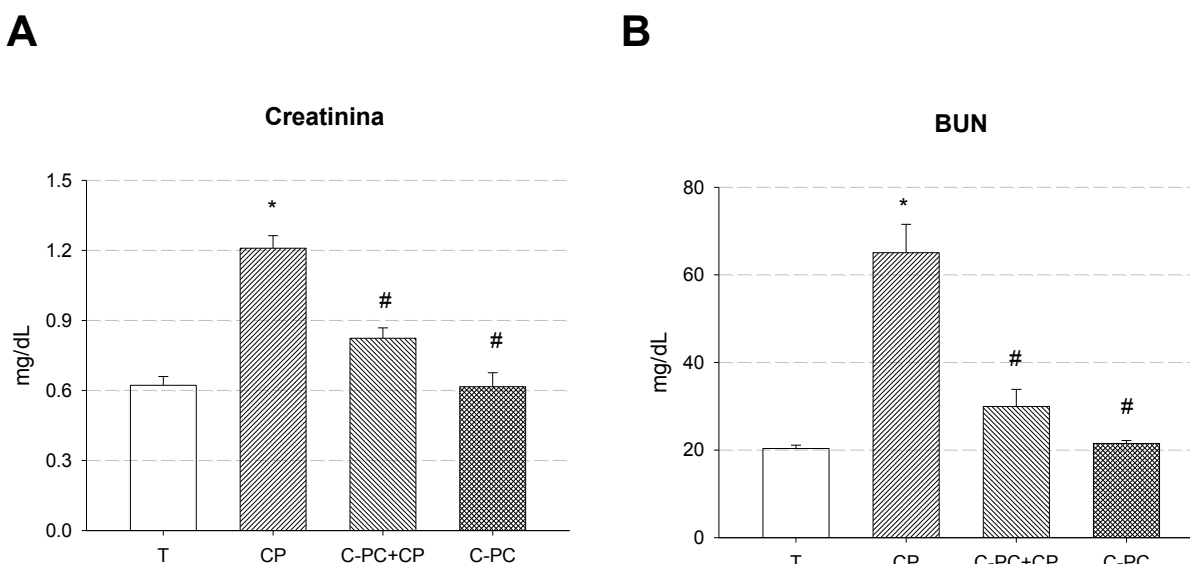


Figura 3A. Marcadores de función renal. La dosis única de CP (22 mg/kg i.p.) incrementó la concentración de creatinina (A) y BUN (B) indicando daño renal. El pre-tratamiento con FC (30 mg/kg i.p.) previno significativamente dicho daño.

12.2.2 Microscopía electrónica de las mitocondrias aisladas

La calidad del método de aislamiento de las mitocondrias se estimó por microscopía electrónica. En la Figura 4A se observan mitocondrias obtenidas a partir de los riñones del grupo T. Las membranas internas y externas están conservadas en la mayoría de los orgánulos estudiados, indicando una alta eficiencia del método de aislamiento de mitocondrias.

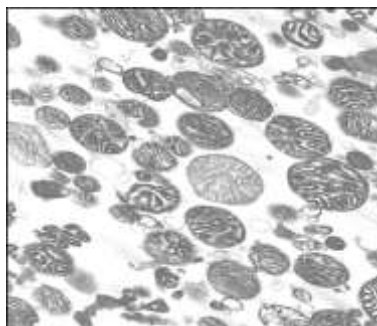


Figura 4A. Microscopía electrónica de mitocondrias aisladas (50,000x). La calidad del aislamiento mitocondrial se evaluó mediante microscopía electrónica. Las mitocondrias están bien conservadas indicando una alta eficiencia en el método de aislamiento.

12.2.3 Oximetría

12.2.3.1 Malato:glutamato como sustrato

En el grupo tratado con CP se redujo marcadamente el estado 3, aumentó el estado 4 y por lo tanto se obtuvo un ICR bajo. La respiración máxima, inducida con FCC, se redujo de manera significativa en comparación al grupo T. En el grupo pre-tratado con FC se previno la reducción del estado 3, más no el incremento del estado 4, además se atenuó la reducción del ICR y la respiración desacoplada inducidos por CP (Figura 5A).

12.2.3.2 Succinato como sustrato

La respiración mitocondrial de los diferentes estados, con succinato como sustrato, fueron significativamente diferentes al grupo T. El grupo de CP redujo la respiración en el estado 3 e incrementó el estado 4. Como consecuencia, disminuyó el ICR (estado 3/ estado 4). La respiración con el desacoplante FCCP, incrementa al máximo la respiración, sin embargo este aumento fue mínimo en el grupo tratado con CP.

En el grupo pre-tratado con FC, se previno la reducción del estado 3 y 4, el ICR y la respiración desacoplada (Figura 6A).

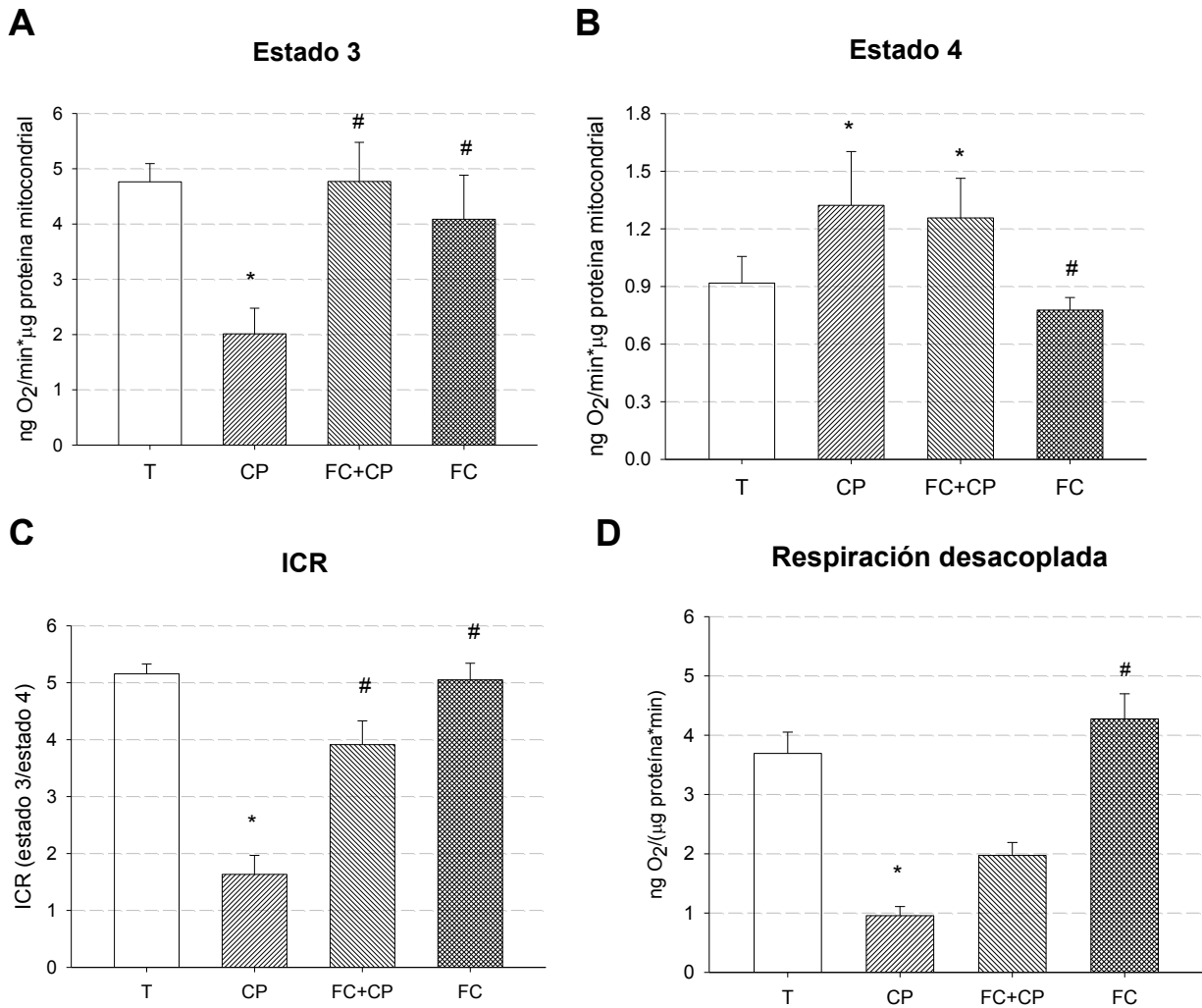


Figura 5A. Respiración mitocondrial con malato/glutamato. En el grupo tratado con CP se reduce significativamente el estado 3 (**A**), se incrementa el estado 4 (**B**) y se reduce tanto el ICR (**C**) como la velocidad máxima (**D**). El grupo pre-tratado con FC evita las alteraciones del estado 3 y el ICR, mas no las del estado 4 ni la respiración desacoplada inducidas por CP. Los datos están expresados como media±E.E., n=3-6. * P<0.05 vs T, # P<0.05 vs CP. T: testigo, CP: cisplatino (22 mg/kg i.p.), FC: ficocianina (30 mg/kg i.p.).

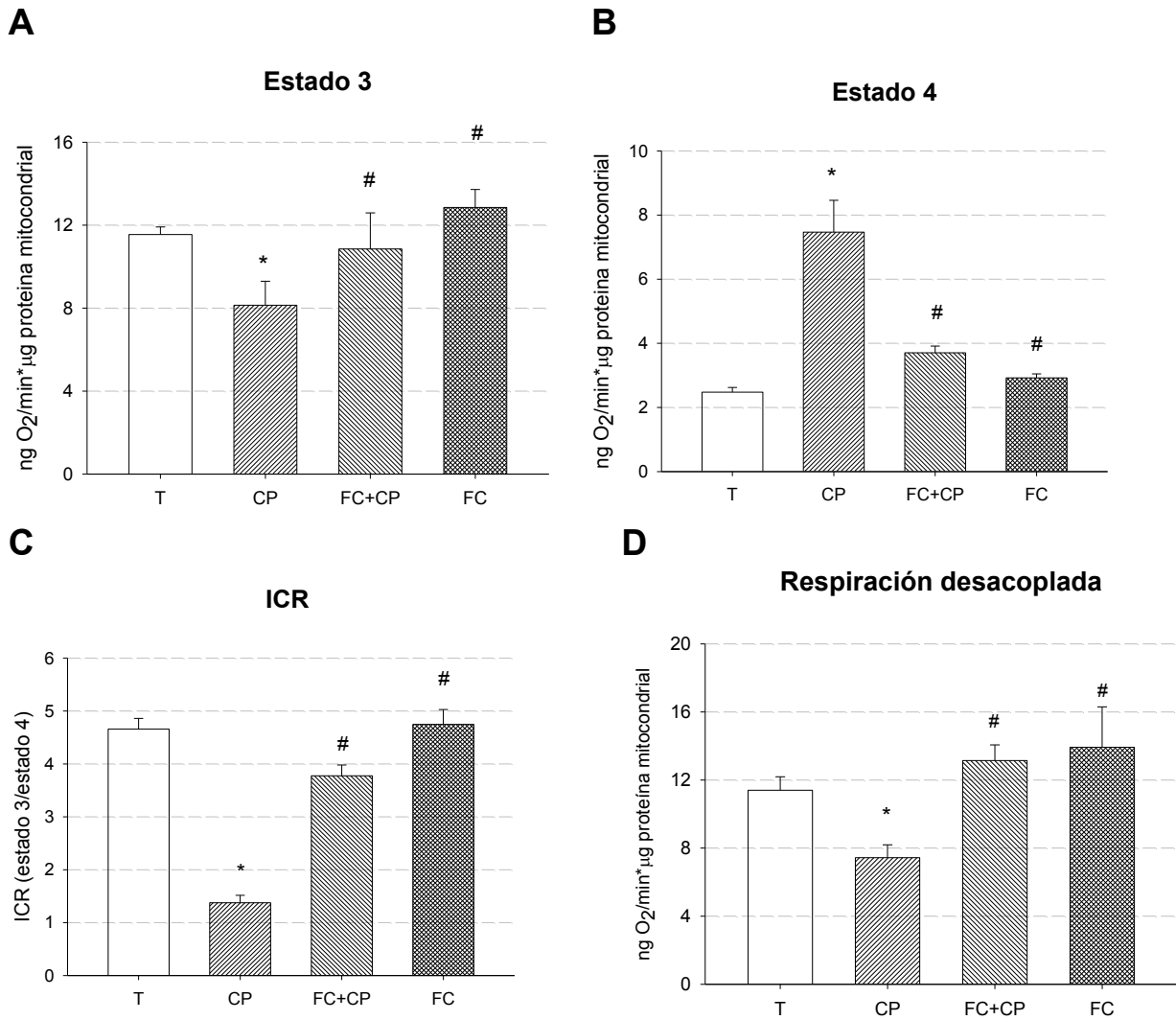


Figura 6A. Respiración mitocondrial dependiente del complejo 2. En el grupo de CP redujo la respiración del estado 3 (ADP) (**A**) e incrementó el estado 4 (sin ADP) (**B**), se reduce el ICR (estado 3/ estado 4) (**C**) y reduce la respiración desacoplada (con FCCP) (**D**). Dichos cambios fueron prevenidos de manera significativa en el tratamiento con FC. Los datos están expresados como media \pm E.E., n=3-6. * P<0.05 vs T, # P<0.05 vs CP, T: testigo, CP: cisplatino (22 mg/kg i.p.), FC: ficocianina (30 mg/kg i.p.).

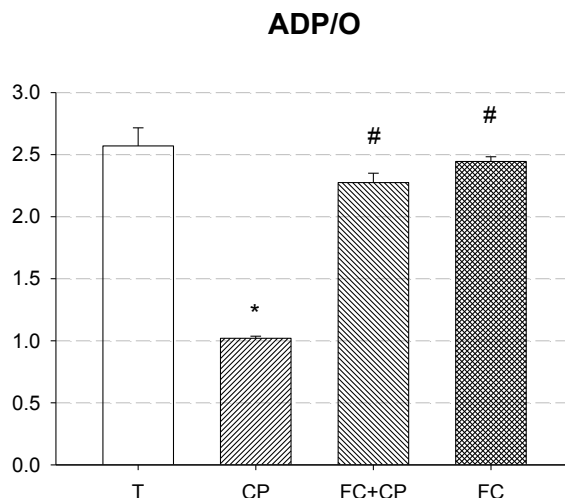


Figura 7A. Efecto de FC en el rendimiento de la síntesis de ATP por molécula de oxígeno consumida durante el estado 3 con malato/glutamato como sustrato. Los datos están expresados como media±E.E., n=4-5. *P<0.05 vs T, # P<0.05 vs CP.

Otro parámetro determinado fue el rendimiento de la síntesis de ATP por consumo de oxígeno (ADP/O) con ambos sustratos (Figuras 7A y 8A). Dicho rendimiento se redujo de manera significativa en el grupo tratado con CP en comparación con el grupo T. Estas alteraciones se previnieron de manera significativa en el grupo FC+CP.

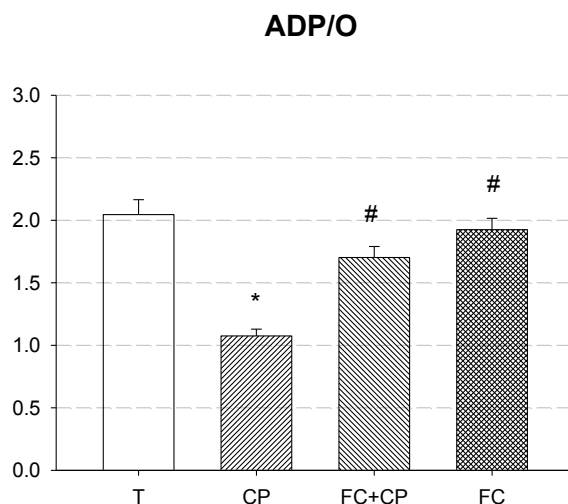


Figura 8A. Efecto de FC en la síntesis de ATP con succinato como sustrato. Relación ADP/O, rendimiento de la síntesis de ATP por molécula de oxígeno consumida durante el estado 3. Los datos están expresados como media±E.E., n=4-5. *P<0.05 vs T, # P<0.05 vs CP.

12.2.4 Síntesis de ATP

El gradiente de protones generados en el transporte de electrones de los complejos mitocondriales I, III y IV se emplea en la síntesis de ATP por el complejo V o ATP sintasa. Los datos de las mitocondrias aisladas de los grupos, revelaron que el grupo tratado con CP reduce marcada y significativamente la síntesis de ATP en comparación al grupo T (Figura 9A).

Estos datos indican que el bombeo de protones por los complejos III y IV probablemente estén reducidos en el grupo tratado con CP aunado a la baja actividad del complejo V, ATP sintasa. Como consecuencia el potencial de membrana generado no es suficiente para sintetizar ATP y/o la ATP sintasa está dañada por CP. El pre-tratamiento con FC, previene la reducción de la síntesis de ATP.

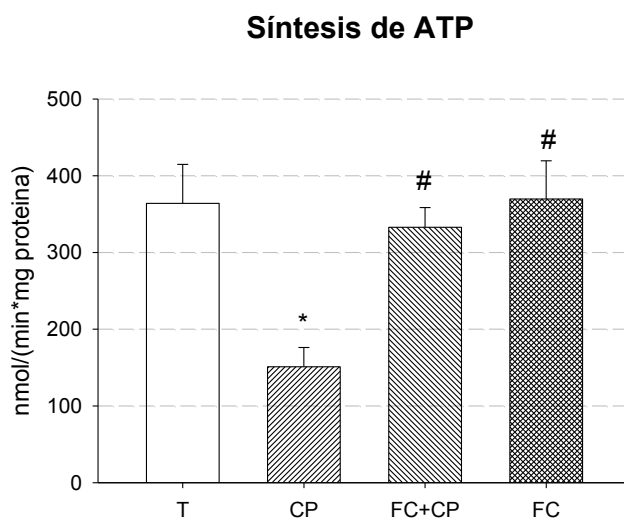


Figura 9A. Síntesis de ATP por la mitocondria con succinato como sustrato. Los datos están expresados como media±E.E, n=4-5. *P<0.05 vs T, # P<0.05 vs CP.

12.2.5 Retención de calcio por la mitocondria

La evaluación del manejo de Ca^{2+} de las mitocondrias de todos los grupos demuestra que el grupo tratado con CP no posee la capacidad de retener el Ca^{2+} adicionado y, tan solo 5 minutos después de añadirlo, las mitocondrias lo liberan al

medio. A diferencia del grupo T, el cual retiene el Ca^{2+} hasta el final del ensayo (Figura 10A). No obstante, el grupo FC+CP previene esta liberación.

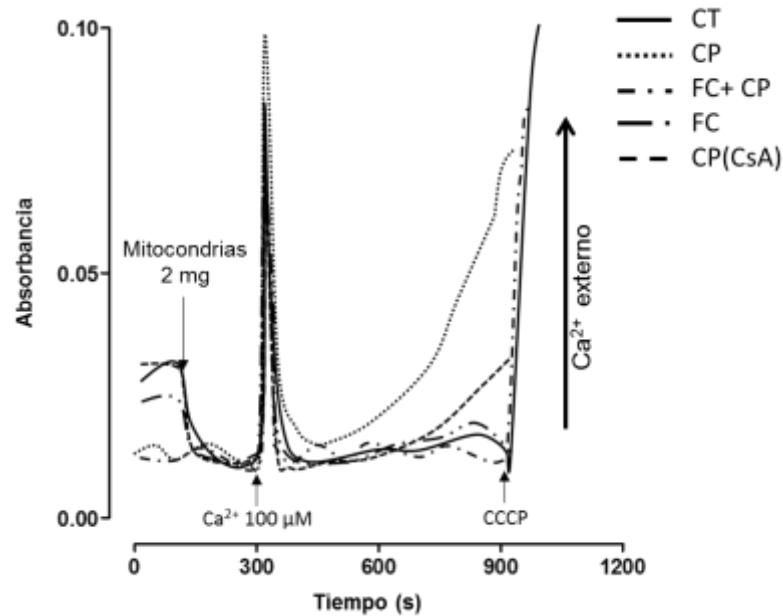


Figura 10A. Efecto de FC en la retención de calcio mitocondrial determinada con arsenazo III. El trazo representa tres experimentos realizados de manera independiente con un pool de 4 ratones por grupo. Ciclosporina (CsA).

12.2.6 Potencial de membrana

Por otra parte, el estudio del potencial de membrana evaluado con safranina, demuestra que el grupo CP pierde el potencial de membrana al poco tiempo de adicionar el Ca^{2+} , mientras que el grupo FC+CP conserva el potencial de membrana por un tiempo más prolongado (Figura 11A).

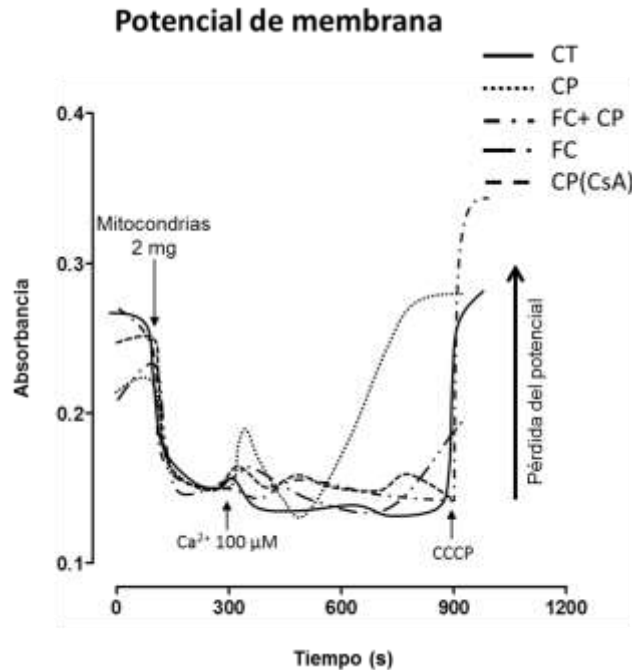


Figura 11A. Trazo representativo del potencial de membrana determinado con safranina de tres experimentos independientes. El grupo CP pierde el potencial de membrana al poco tiempo de adicionar Ca^{2+} 100 mM a los 600 s.

12.2.7 Daño oxidante mitocondrial

El cisplatino indujo daño oxidante en las mitocondrias renales aisladas y redujo la concentración de GSH de manera significativa con respecto al grupo T. A su vez, redujo la actividad de la enzima aconitasa, la cual es muy sensible al daño oxidante. En el grupo pre-tratado con FC, se redujo la pérdida del contenido de GSH y se atenúo la pérdida en la actividad de dicha enzima (Figura 12A).

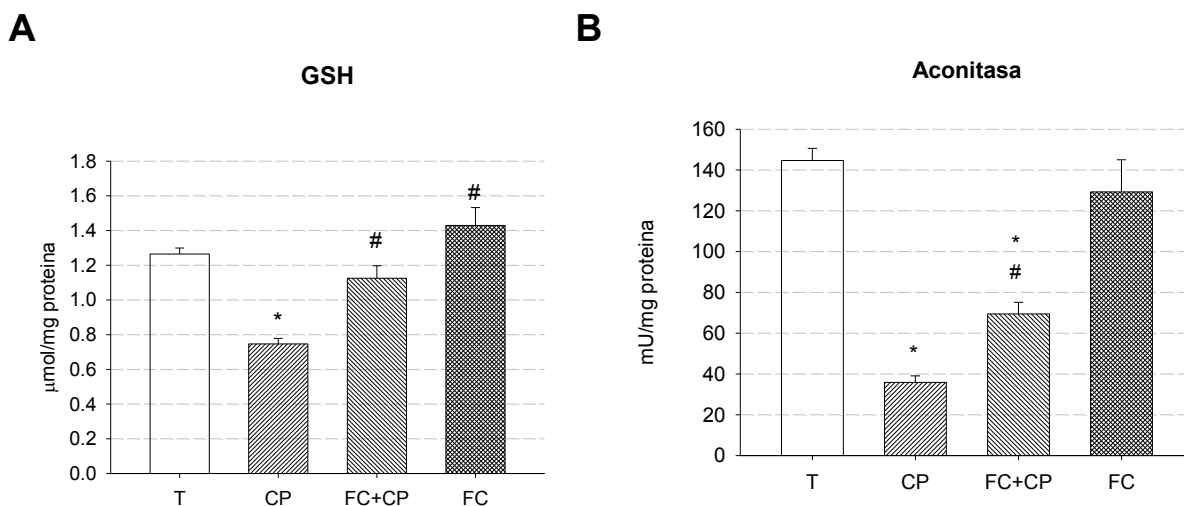


Figura 12A. Marcadores de daño oxidante mitocondrial. El pre-tratamiento de FC previene la reducción de **(A)** GSH y **(B)** de la actividad de aconitasa inducida por CP. Los datos están expresados como media±E.E., n=3-6. *P<0.05 vs T, # P<0.05 vs CP. T: testigo, CP: cisplatino (22 mg/kg i.p.), FC: ficocianina (30 mg/kg i.p.), GSH: glutatión.

12.2.8 Actividad antioxidante mitocondrial

Se evaluó la actividad antioxidante en las mitocondrias renales aisladas de los diferentes grupos. La FC previno significativamente la reducción en la actividad de CAT y atenuó la reducción en la actividad de GPx y GR inducidos por CP. La actividad de SOD solo fue ligeramente reducida en el grupo tratado con CP. La FC *per se* no modificó la actividad de GPx, CAT y SOD pero aumentó la actividad de GR (Figura 13A).

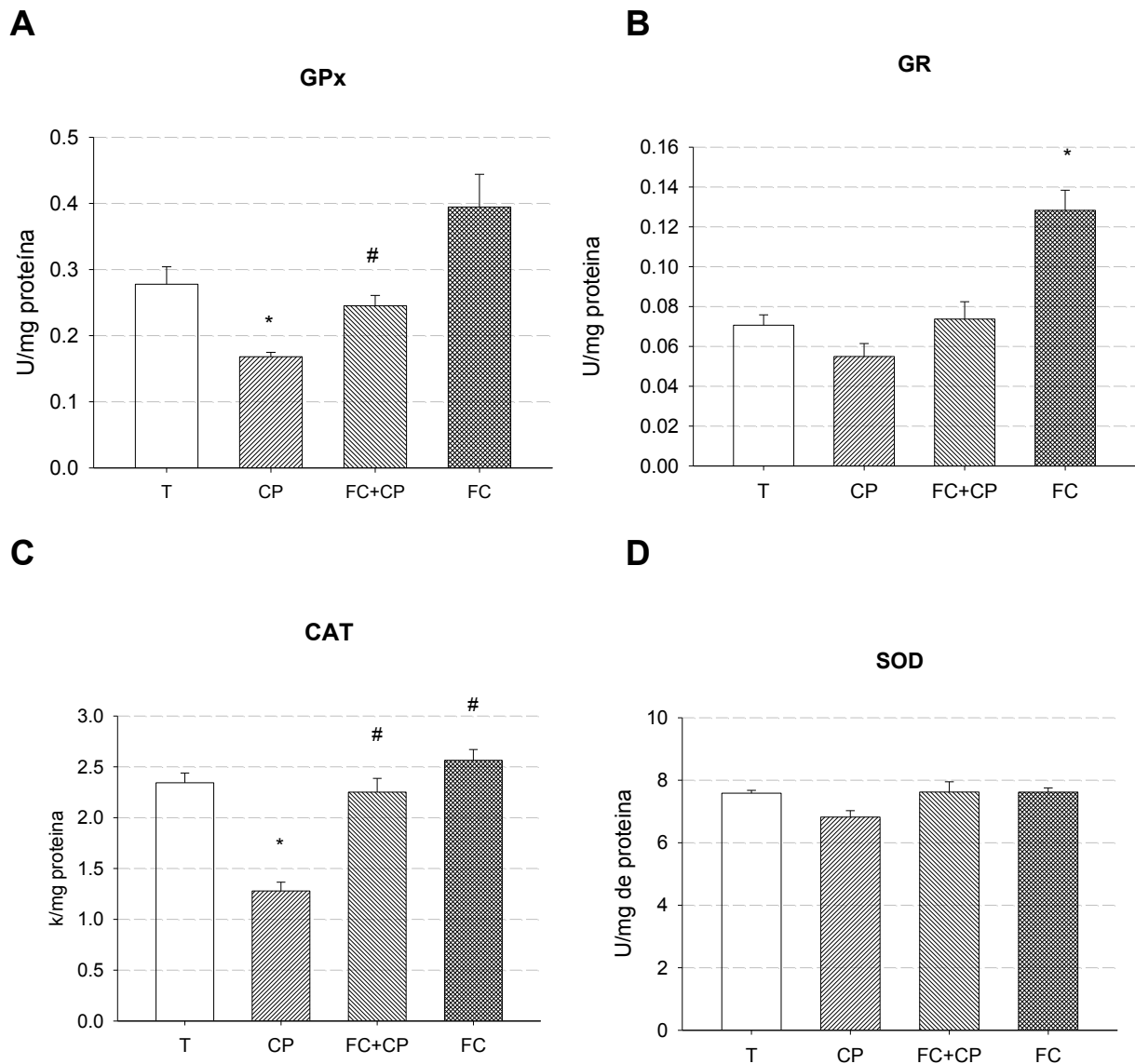


Figura 13A. Actividad antioxidante mitocondrial. El grupo CP redujo significativamente la actividad de **A:** GPx y **C:** CAT. Atenúo la actividad de **B:** GR y **C:** SOD. El grupo FC+CP previno la reducción de la actividad de CAT y atenúo las alteraciones en GPx y GR. Los datos están expresados como media±E.E., n=3-6. * P<0.05 vs T, # P<0.05 vs CP, T: testigo, CP: cisplatino (22 mg/kg i.p.), FC: ficocianina (30 mg/kg i.p.), GPx: glutación peroxidasa, CAT: catalasa, GR: glutación reductasa, SOD: superóxido dismutasa.

13 ARTÍCULOS GENERADOS

13.1 Nutreceptual properties of phycocyanin

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Nutreceptual properties of phycocyanin



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ABSTRACT

Phycocyanin (PC) is one of the main pigments of the algae *Spirulina*, which is used as a dietary supplement due to its high protein content. PC is a protein from the phycobiliprotein family characterized by its intense blue color and its structure consists of a protein and non-protein components known as phycocyanobilin. PC scavenges reactive oxygen and nitrogen species (ROS and RNS, respectively) and prevents oxidative damage that may explain, at least in part, its beneficial effects. This review focuses on the beneficial characteristics and properties of PC emphasizing the antioxidant activity on *in vitro* and *in vivo* models. The use of PC in clinical trials is warranted.

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1. Introduction

Recently, many scientific papers have focused their attention on health promoting properties of several foods (Avila-Nava et al., 2014; Braithwaite et al., 2014; Tahergorabi, Matak, & Jaczynski, 2014). The role of functional foods and their components in disease risk reduction, treatment and promotion of human health has been demonstrated (Griffiths, Abernethy, Schuber, & Williams, 2009; Lobo, Patil, Phatak, & Chandra, 2010; Tenore, Novelino, & Basile, 2012; Vučić et al., 2014; Zhuang, Tung, & Yuan, 2013). Functional foods contain one or more bioactive(s) and as such nutraceutical are defined as compounds or products that have been isolated or purified from food sources that possess demonstrated health-promoting properties (Custódio et al., 2008; Lattanzio, Kreon, Linsalata, & Cardinali, 2009; Roberfroid, 2000). In this regard, phycocyanin (PC) is a nutraceutical compound with biological activity isolated and/or purified from seaweeds (De Jesus Raposo, de Moraes, & de Moraes, 2013; Pangestani & Kim, 2011). PC has shown anti-inflammatory, antiplatelet, anti-cancer, nephroprotective and hepatoprotective properties that may be explained, at least in part, by its antioxidant activity. The aim of this review is to critically analyze the properties of PC and the results obtained from studies that show the involvement of the antioxidant activity of this protein in its nutraceutical properties.

2. General characteristics

PC is a protein from the phycobiliprotein (PBP) family (Patel, Mishra, Pawar, & Ghosh, 2005) characterized by its intense blue color. It is a peripheral accessory light-harvesting complex called phycobilisome (PBS), which is assembled on the surface of the thylakoid membrane. Its main function is to transfer the excitation energy to the center reaction where the maximum wavelength of absorption is near to 620 nm (Benedetti et al., 2006; De Marsac & Cohen-Bazire, 1977).

PC is one of the main pigments of Mexican algae *Spirulina*, which is used as a dietary supplement due to its high content of protein, vitamins, minerals and essential fatty acids (Alman, Mashuda, Tam, & Mohammad, 2008; Cherng, Cheng, Tarn, & Chou, 2007; Manconi et al., 2009; Thanh-Sang, BoMi, &

Se-Kwon, 2013). This pigment is found in cyanobacteria and eukaryotic algae such as Rhodophyta and Cryptomonads (Glazer & Stryer, 1983). PC is classified into three types, C-PC (obtained from cyanobacteria), R-PC (obtained from red algae) and R-PCII (obtained from *Synechococcus* species) (Kuddus, Singh, Thomas, & Al-Hazimi, 2013; Wang et al., 2014). In this review, the general properties of the three types of PC are described. PC has been extracted from different algae sources (Table 1). In the *Spirulina* algae, PC provides its characteristic green-blue color (Eriksen, 2008; Gantar, Simović, Djilas, Gonzalez, & Miksovská, 2012; Silveira, Quines, Burkert, & Kalil, 2008).

Furthermore, PBPs are the most abundant proteins in many cyanobacteria and algae. However, it has been postulated that PBPs are not essential for the cell function because they are degraded when nitrogen is deprived. Therefore, PBPs are considered as a source of nitrogen storage (Sloth, Wiebe, & Eriksen, 2006).

PBPs are water soluble; they have bright color and are highly fluorescent. Also, they exhibit different and unique qualitative and quantitative features including a broad spectrum of visible light absorption and high absorption coefficient (Chattopadhyay et al., 2012; Glazer & Stryer, 1983).

PC purity is evaluated based on the absorbance ratio A620/A280. The absorbance at 620 and 280 nm correspond to PC and total protein, respectively (Patil, Chethana, Sridevi, & Raghavarao, 2006). PC is considered food grade when A620/A280 is 50.7, reagent grade when A620/A280 is between 0.7 and 3.9 and analytical grade when A620/A280 is 24.0 (Antelo, Anschau, Coets, & Kalil, 2010; Kuddus et al., 2013; Patil et al., 2006).

Equation (1) is used to determine the PC concentration (mg/ml) in crude extracts (Antelo et al., 2010; Bennett & Rogorad, 1973; Patel et al., 2005; Silveira et al., 2008).

$$[\text{PC}] = \frac{O.D_{620\text{nm}} - 0.474(O.D_{620\text{nm}})}{5.34} \quad (1)$$

The optical density at 652 nm corresponds to allophycocyanin; another PBP. The lethal dose 50 (LD₅₀) of PC analytic grade has not been established yet. The higher dose used with no observed adverse effect level (NOAEL) was studied by Romay, Ledón, and González (1998b); they found a NOAEL of 3 g/kg. Currently it has been shown that NOAEL of PC given orally is 3 g/kg (Ou, Lin, Pan, Yang, & Cheng, 2013). The NOAEL of

Table 1 – Algae sources from which phycocyanin has been purified.

Algae	Reference
<i>Acaryochloris marina</i>	Samsonoff and MacColl (2001)
<i>Anabaena</i>	Gentler et al. (2012)
<i>Anabaena flos-aquae</i>	Shanab, Mostafa, Shalsby, and Mahmoud (2012)
<i>Anabaena variabilis</i>	Chapman, Cole, and Siegelman (1968)
<i>Anacystis nidulans</i>	Chapman et al. (1968)
<i>Aphanizomenon</i>	Gentler et al. (2012)
<i>Aphanizomenon flos-aquae</i>	Rinalducci, Roepstorff, and Zolla (2009)
<i>Arthrospira africana</i>	Minkova et al. (2002)
<i>Arthrospira (Spirulina) maxima</i>	Manonnis et al. (2008); Rodriguez-Sánchez et al. (2012); Ou et al. (2010)
<i>Arthrospira fusiformis</i> (Voronch) strain Hegewald 1976/83	Ivanova et al. (2010)
<i>Calothrix membranacea</i>	Chapman et al. (1968)
Cryptomonads	Samsonoff and MacColl (2001)
<i>Cyanidium caldarium</i>	Samsonoff and MacColl (2001)
<i>Democorys violacea</i>	Chapman et al. (1968); Samsonoff and MacColl (2001)
<i>Galderia sulphurata</i>	Sethi et al. (2006)
<i>Limnolthrix</i>	Gentler et al. (2012)
<i>Limnolthrix</i> sp. strain 37-2-1	Gentler et al. (2012)
LSD 0.05	Shanab et al. (2012)
<i>Lyngbya</i> sp.	Chapman et al. (1968); Gentler et al. (2012); Patel et al. (2005); Patel, Sandhya, and Ghosh (2006)
<i>Marthococcus laminosus</i>	
<i>Microcystis aeruginosa</i>	Chapman et al. (1968)
<i>Nostoc</i>	Shanab et al. (2012)
<i>Nostoc muscorum</i>	Gentler et al. (2012)
<i>Oscillatoria lewisii</i>	Chapman et al. (1968); Shanab et al. (2012); Shukla, Singh, Kashyap, Giri, and Kashyap (2008); Srivastava (2010)
<i>Phormidium biguttulatum</i>	Thangum et al. (2013)
<i>Phormidium fragile</i>	Kumar and Gaur (2014)
<i>Phormidium</i> sp.	Shanab et al. (2012); Sen, Trivedi, and Madamwar (2008)
<i>Phormidium luridum</i>	Patel et al. (2005); Patel, Pawar, Mishra, Sonawane, and Ghosh (2004); Patel et al. (2008); Shukla et al. (2008)
<i>Plectononema boryanum</i>	Chapman et al. (1968)
<i>Porphyra tenera</i>	Chapman et al. (1968)
<i>Porphyra yezoensis</i> Ueda	Chuner et al. (2011)
<i>Porphyra yezoensis</i>	Chuner et al. (2011)
<i>Polysiphonia uccoleta</i>	Wang et al. (2014)
<i>Smithora nasutum</i> var. <i>Nasutum</i>	Chapman et al. (1968)
<i>Spirulina</i> sp.	Patel et al. (2004); Patel et al. (2005); Patel et al. (2006); Li, Chen, Wong, Xu, and Fan (2011); Wu et al. (2011); Bernejo et al. (2008); Chu, Lim, Radhakrishnan, and Lim (2010); Li et al. (2010); Marin-Frutos et al. (2013); Ou et al. (2012); Fenton-Kol et al. (2012a); Flenoud et al. (2013); Zhang et al. (2011); Zheng et al. (2014)
<i>Spirulina (Arthrospira) fusiformis</i>	Minkova et al. (2002); Madhyastha and Vatsala (2010)
<i>Synechococcus</i> sp. 10S201	Abeliov (1998)
<i>Synechococcus</i> s	Gentler et al. (2012)
<i>Synechococcus</i> p. (strain 8301)	Glazer, Cohen-Bazire, and Jui (1971)
<i>Synechococcus lundii</i>	Chapman et al. (1968)
<i>Wetmorella</i> sp.	Sabarnathan and Ganesan (2005)
<i>Wollea sacata</i>	Shanab et al. (2012)

70 mg/kg by intraperitoneal (i.p.) route was used in rats, showing no side effects (Gupta, Dwivedi, & Khandelwal, 2011). However, the higher dose of 200 mg/kg i.p. has been used in experiments with Wistar rats (González et al., 2003; Kumari & Anbarasu, 2014; Vadiraja, Gaikwad, & Madhyastha, 1998). PC is classified in category 3 according to the International Labor Organization and based on the oral dose of 5 g/kg. Additional research with higher doses, given orally or i.p., of analytical grade PC is needed to definitively establish this compound as non-toxic. Further information about the metabolites produced and/or generated by different routes of administration is required because it is an issue that has not yet been explored.

PC food grade is generally recognized as safe (GRAS) food used as dye in the formulation of food products such as desserts, sweets, cake decoration, milkshakes, gum, jellies and ice cream as well in cosmetics, alcoholic beverages, biotechnology and in medicine (drugs) (Antelo et al., 2010; FDA, 2012; Kim, Ravichandran, Khan, & Kim, 2008; Yoshida, Takagaki, & Nishimune, 1996).

The isoelectric point of PC varies between 4.1 and 6.4 depending on the algae source and methods employed for extraction and purification (Chen & Wong, 2008; Silveira et al., 2008; Wang et al., 2014); its extinction coefficient ($\epsilon^{1\%}_{1\text{cm}}$) at 620 nm is of $770,000 \text{ M}^{-1}\text{cm}^{-1}$ (Senedetti et al., 2004). Formerly, it was believed that PC was not light sensitive (Abeliovich & Shilo,

1972) but recent evidences indicate that it is light sensitive and must be kept in darkness (Benedetti et al., 2006; Wang et al., 2014). In fact, PC produces reactive oxygen species (ROS) under light conditions as it is described later. Moreover, this protein must be purified between 4 and 5 °C (Benedetti et al., 2006; Niu, Wang, Liu, & Zhou, 2007; Fatil, Chethana, Madhusudhan, & Raghavarao, 2008) because it is heat sensitive (Benedetti et al., 2006; Chaiklahan, Chiratrivan, Loha, Tia, & Bunnag, 2011; Oliveira, Rosa, Moraes, & Faria, 2009; Silveira et al., 2008), besides its purity decreases through the time (Gantar et al., 2012).

3. Fluorescence

PBSs are disintegrated when they are extracted in buffer, as a result, the PFBs lose their capacity to transfer electrons in the photosynthetic process conferring to PFBs a fluorescent property (Eriksen, 2002). Compared to other fluorophores, PFBs have a high molar extinction coefficient and high fluorescence quantum yield. The PC maximum excitation wavelength is close to 620 nm with an emission peak at 640 nm.

PFBs are conjugated to immunoglobulins, protein A and avidin. This property has been used for histological applications, fluorescence microscopy, immunoassays and fluorescence-activated cell sorting. However, PC loses their fluorescence and absorbance when is denatured.

4. PC structure

PC is composed of a protein and a non-protein component known as phycocyanobilin (PCB). Figure 1 shows how PFBs and their complex form the PBS located at the thylakoid outer membrane; they are adjacent to the reaction center of photosystem II light harvesting apparatus (Patel et al., 2005). Its main function is to collect light efficiently when chlorophyll absorbs and transfer poorly the light energy to chlorophyll in the thylakoid membrane (Liron et al., 2014; McColl, 2004; Zilinskas & Greenwald, 1986). The structure of PCB is similar to that of bilirubin and biliverdin (Fig. 2) (Lakshmi, Maheswari, & Annamalai, 2008). PCB is an open-chain tetrapyrrole responsible for the intense blue color of PC.

The protein component of PC consists of two homologous subunits, α and β chain types globin covalently linked to type PCB by thioether linkage (Fig. 3).

According to Scheer and Zhao (2008), α chain is attached to one PCB via cysteine 84 and β chain is joined to two PCBs via cysteines 84 and 153 by thioether linkages. In contrast, Tang et al. (2012) described that it is bound to the cysteine 153 and Shen et al. (2008) suggested that PCBs are bound to the cysteines 82 and 153 (Shen et al., 2006; Shen, Schlachter, & Bryant, 2008). Both chains have α -helix structure and the three-dimensional structures are similar among all organisms (Scheer & Zhao, 2008). According to its primary structure, α subunit has two cysteines and two methionine residues. The β subunit contains three cysteines and five methionine residues (Huang, Guo, Wong, & Jiang, 2007).

X-ray crystallographic analyses of PC have shown that the chiral C3 carbons of the PCB chromophores attached to cys-

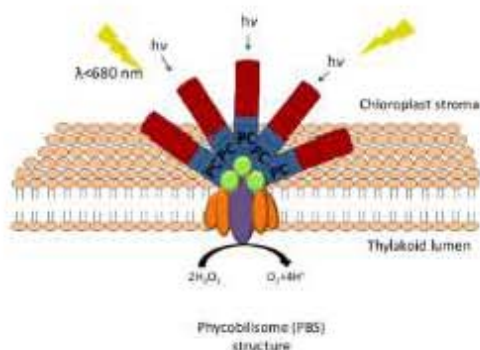


Fig. 1 – Structure of phycobilisome (PBS), the major complex light collection in cyanobacteria and red algae.

PBSs are composed of hundreds of seemingly similar chromophores, which are proteins. They are assembled in a fashion that enables highly efficient transfer of energy unidirectionally to the center reaction. Several phycobiliproteins (PFBs) are involved in a cascade of energy transfer from phycoerythrin (PE, red cylinders) to phycocyanin (PC, blue cylinders) and allophycocyanin (APC, green sphere) and finally to the reaction center in photosystems II (purple ellipse) and I (orange ellipses). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

teines α 84 and β 82 are in the R configuration, whereas the C3 carbon of the PCB at cysteine β 153 is in the S configuration (Schirmer, Bode, Huber, Sudler, & Zuber, 1985; Shen et al., 2006).

The monomer of PC is formed by the α and β chains, these form aggregates naturally as trimer ($\alpha\alpha\beta$) or hexamer ($\alpha\alpha\beta\beta$), the latter is the functional form of PC and usually forms blocks into the antenna complex (Padyana, Bhat, Madyastha, Rajashankar, & Ramakumar, 2001; Scheer & Zhao, 2008). The $\alpha\beta$ is composed of nine PCB units assembled by three equal parts (Womick & Moran, 2009). The molecular weight of subunits varies depending on the algae source and methods for extraction and purification. The α subunit is between 13 and 20.5 kDa and β subunit is between 11 and 24.4 kDa. The protein Data Bank describes that the molecular weight of PC obtained from *Spirulina maxima* is 121 kDa ($\alpha\beta$); the molecular weight for the α -chain is 17,302.6 Da and for the β -chain is 17,827.2 Da (Satyanarayana, Patel, Mishra, Ghosh, & Suresh, 2007). According to the database, the apoprotein contains 20 amino acids and provides the fluorescent activity of PC. Besides, it is able to chelate Fe^{3+} and Hg^{2+} (Liron et al., 2014; Suresh, Mishra, Mishra, & Das, 2009). PC trimers have a disk shape of a diameter of 11 nm, a thickness of 3 nm and a cavity of 3.5 nm in diameter at the center (Sun, Wang, & Qiao, 2006).

5. PC as an antioxidant

It is well established that antioxidants are able to prevent ROS production and/or to scavenge them (Halliwell, 1996;

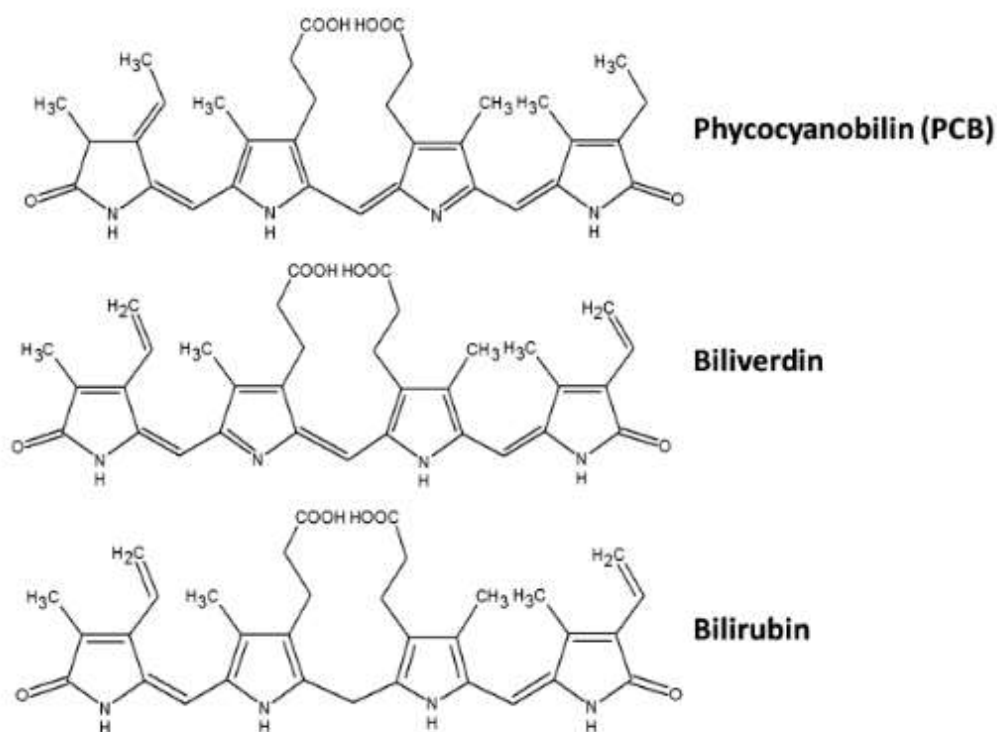


Fig. 2 – Chemical structure of phycocyanobilin (PCB), biliverdin and bilirubin. PCB is an open tetrapyrrole similar to biliverdin and bilirubin structure.

Samaransyaka & Li-Chan, 2011; Šliumpaitė, Venskutonis, Murkovic, & Fukalskas, 2013; Wiriyaphan, Chitsomboon, Roytrakul, & Yongswadigul, 2013). The imbalance between antioxidant systems and ROS production is called oxidative stress. This latter is related to various diseases including atherosclerosis, neurodegenerative diseases, cancer, diabetes mellitus, inflammatory diseases, as well as aging processes (Durancková, 2010).

In 1998, Cuban researchers described for the first time the antioxidant activity of PC (Romay et al., 1998a). These authors determined that PC was able to scavenge hydroxyl radical ($^{\bullet}\text{OH}$), alkoxyl radical (RO^{\bullet}) and superoxide anion ($\text{O}_2^{\bullet-}$). PC was also able to inhibit lipid peroxidation (Romay et al., 1998a). In addition they described for the first time the anti-inflammatory properties of PC (Romay et al., 1998b). It took a little longer for other researchers worldwide to confirm previous results or to discover that PC scavenged singlet oxygen ($^1\text{O}_2$) (Tapia, Galetovic, Lerpmp, Pino, & Eduardo, 1999), hypochlorous acid (HOCl) (Bermejo, Finero, & Villar, 2008; Romay, Gonzalez, Pizarro, & Lissi, 2000), peroxy radical (ROO^{\bullet}) (Benedetti, Benvenuti, Scoglio, & Canestrari, 2010; Romay et al., 2000), $^{\bullet}\text{OH}$ (Bermejo et al., 2008), peroxynitrite (ONOO^{\bullet}) (Bhat & Madyastha, 2001), nitric oxide (NO) (Thangam et al., 2013) and hydrogen peroxide (H_2O_2)

(Fernandez-Rojas et al., 2014) (Fig. 4). PC is also able to scavenge the non-natural radicals 2,2-azino-bis(3-ethylbenzothiazolin-6-sulphonic acid) diammonium salt ($\text{ABTS}^{\bullet+}$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Chen & Wong, 2008; Huang et al., 2007). The $\text{O}_2^{\bullet-}$, $^{\bullet}\text{OH}$, DPPH and $\text{ABTS}^{\bullet+}$ scavenging activity of the purified PC from selenium-enriched *Spirulina platensis* algae (PC-Se) was slightly increased (Chen & Wong, 2008; Huang et al., 2007).

Currently, some mechanisms have been proposed to explain how PC stabilizes ROS. Both components are involved, the apoprotein (α and β subunits) and PCB (Pleasant, Soogarun, & Suwanwong, 2013).

Apoprotein has been separated using methanol and denaturing agents such as sodium dodecyl sulphate (SDS), urea and trypsin. These agents have been used to demonstrate that apoprotein contributes to the $^{\bullet}\text{OH}$ scavenging activity. In fact, a change in pH from 7 to 11 increased the $^{\bullet}\text{OH}$ scavenging activity because the changes in protein charge modified its configuration (Huang et al., 2007). Romay et al. (2000) reported that apoprotein scavenges HOCl by reacting with cysteine and methionine residues. Other amino acids such as tryptophan, tyrosine and histidine were able to scavenge ROO^{\bullet} (Fateh et al., 2005).

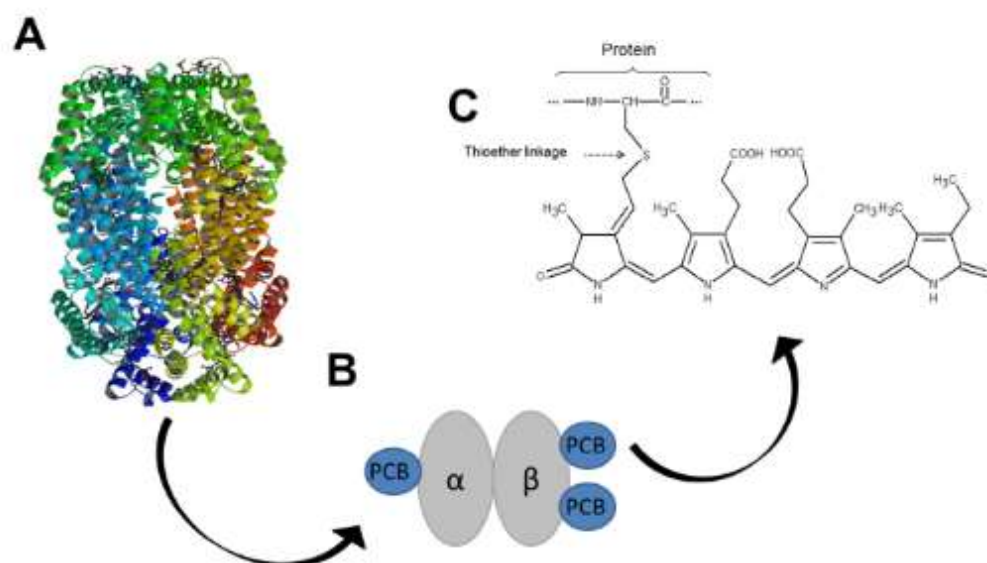


Fig. 3 – Phycocyanin (PC) structure. (A) Crystal structure of PC from cyanobacterium *S. platensis* in form of hexamer, image from PDB (<http://www.rcsb.org>) of PDB ID 1GH0 (2011). (B) Schematic representation of PC assembly. It is composed of two protein subunits, α and β chains, one phycocyanobilin (PCB) is bound to the α subunit and two PCBs are bound to the β subunit. (C) Chemical structure of PCB, the chromogen responsible of blue color of PC.

PCB scavenges most radicals (Fleonsil et al., 2013; Zhan-Ping et al., 2005). 1O_2 is stabilized by oxidation of double bonds of the tetrapyrrole (Romay et al., 2000). PCB also scavenges ONOO (Bhat & Madyastha, 2001), HOCl (Lissi, Pizarro, Asper, & Romay, 2000), $^{\bullet}OH$ (Phuang et al., 2007) and ROO^{\bullet} (Patel et al., 2005). Other assays have been used to determine the scavenging activity of PC including oxygen radical absorbance capacity (ORAC), ferrous ion oxidation xylenol orange (FOX), ferric reducing ability of plasma (FRAP) assay, reducing power assay and intracellular reactive oxygen levels using 2',7' dichlorofluorescein diacetate (DCFH-DA) (Madhyastha, Svashankari, & Vatsala, 2009). During the scavenging activity of PC, its color gradually fades and its absorbance at 620 nm is reduced, its fluorometric intensity disappears and two new bands are observed at approximately 640 nm (Benedetti et al., 2004; Lissi et al., 2000; Patel et al., 2005; Tapia et al., 1999).

Under different light conditions PC has a bi-functional activity, it can generate $^{\bullet}OH$, whereas in the dark it traps them. The ROS production is counteracted when concentration of PC increased (Zhan-Ping et al., 2005). However, Madhyastha et al. (2009) have shown that blue light increases the scavenging activity against DPPH, $^{\bullet}OH$ and HOCl as well as in the ORAC and FRAP assays due to the induction of a conformational change in α and β chains altering the presence of cysteines (Madhyastha et al., 2009).

Once the antioxidant properties of PC were demonstrated; this protein was subjected to several oxidizing conditions to evaluate its biological efficacy against some pathological disorder in *in vitro* and in *in vivo* models. Currently, it is well

established that PC is able to trap several ROS. Besides, PC may prevent the development of some diseases in which oxidative stress is involved. The results obtained from these *in vivo* and *in vitro* studies will be discussed later.

6. In vitro studies

Several studies have evaluated the effect of PC in cancer cells and in non-cancer cells from several organs under different protocols and oxidative challenges (Table 2, Table 3 and Fig. 5).

6.1. Cancer cells

PC-Se has shown to prevent the cellular proliferation of human melanoma A375 cells and human breast adenocarcinoma MCF-7 cells by the induction of the intrinsic pathway of apoptosis (Table 2). This latter was characterized by a reduction in the mitochondrial membrane potential in cancer cells but not in normal human fibroblast Hs68 cells (Chen & Wong, 2008, Fig. 5A). Therefore, these data suggest the use of PC as a prophylactic agent for cancer diseases.

On the other hand, PC showed beneficial effects in terms of drug resistance. It is known that the expression of multidrug resistance protein (MDR-1), involved in the resistance of distinct anticancer drugs such as doxorubicin (DOX), is regulated by ROS and cyclooxygenase-2 (COX-2) expression in human hepatocellular carcinoma (HepG2) cells. As described earlier, PC

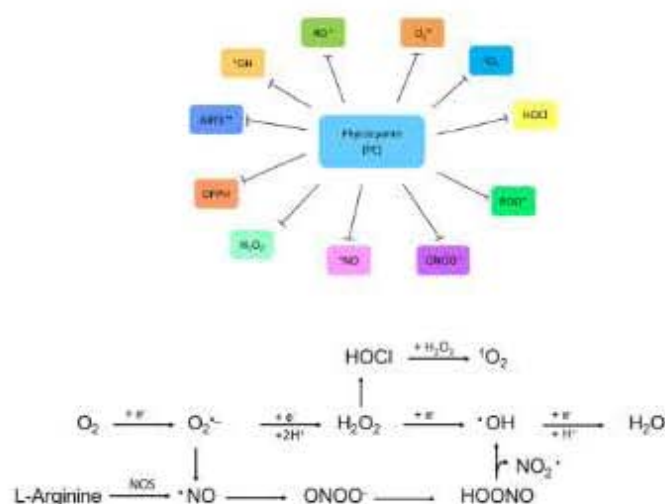


Fig. 4 – Phycocyanin scavenges several reactive oxygen species (ROS). The sequential univalent reduction of oxygen is shown in the lower panel. PC is able to scavenge all ROS tested in different studies. •OH, hydroxyl radical; RO•, alkoxy radical; O₂^{•-}, superoxide anion; ¹O₂, singlet oxygen; HOCl, hypochlorous acid; ROO•, peroxy radical; ONOO•, peroxyntrite; •NO, nitric oxide; H₂O₂, hydrogen peroxide; APTS^{••}, 2,2'-azinobis(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

may prevent MDR-1 induction because it is able to scavenge ROS and inhibit COX-2 expression. In fact, PC prevents the resistance to DOX in HepG2 cells (Nishanth et al., 2010, Fig. 5A). Interestingly, does not interfere with the antineoplastic activity of DOX in human ovarian cancer cells (Khan et al., 2006b).

The use of PC has been proposed as a photosensitizer in HepG2 cells when these are pre-treated with PC and irradiated with laser (He-Ne). These cells initiate the apoptotic process through the intrinsic pathway (Wang et al., 2012); PC also induces the loss of mitochondrial membrane potential, the release of cytochrome c by the ROS increase, the activation of caspase-3 and the arrest of the cell cycle in G2/M, but not in the normal human liver cell line HL7702 (Wang et al., 2012, Fig. 5). When confocal microscopy was used, PC was located

inside mitochondria (Wang et al., 2012), which may explain its apoptotic effects.

In colon cancer HT-29 cells and lung adenocarcinoma A549 cells, PC induced, in a dose dependent manner, a reduction in cell viability by inducing apoptosis. In addition, other authors have previously described that PC treatment increases nuclear condensation in cancer cells in a dose-dependent manner. Also, DNA fragmentation increases and cells are arrested at the G0/G1 phase of cell cycle, probably as a checkpoint to prevent cell replication (Thangam et al., 2013).

Finally, the use of PC has been proposed as an agent in the photodynamic therapy that in combination with He-Ne light could provide a possible tumor therapy (Li, Chu, Gao, & Li, 2010; Wang et al., 2012). Indeed, PC has been suggested

Table 2 – Anticancer properties of PC on in vitro studies.

Cell line	PC concentration	Major outcomes	Reference
HT-29 A549	5–60 µg/ml, for 48 h 30 µg/ml, (IC ₅₀) 55 µg/ml, (IC ₅₀)	Antioxidant and antiproliferative activity, apoptosis, membrane blebbing, nuclear condensation and DNA fragmentation.	Thangam et al. (2013)
HepG2	Co-treatment PC (1–100 µM) and DOX (100 nM–50 µM) for 12–48 h.	The expression of MDR-1 is regulated by inhibition of ROS and COX-2.	Nishanth et al. (2010)
MCF-7 A575	0–40 µM 5e-PC for 72 h.	Antiproliferative and apoptotic effect. Disruption of mitochondrial membrane potential.	Chen and Wang (2006)
HepG2	Pre-treatment for 4 h with PC (0–400 µg/ml) and exposed to He-Ne (26 J/cm ²) laser beam. Cultured for 24 h. 100 µg/ml, (IC ₅₀)	Apoptosis is induced by high levels of ROS, causing mitochondrial damage, 1 µM, release cytochrome c and caspase 3 activation.	Wang et al. (2012)

IC₅₀: inhibitory concentration, MDR: multidrug resistance protein, COX-2: cyclooxygenase-2, 1 µM: loss of mitochondrial membrane potential.

Table 3 – Antioxidant protective effect of PC in several *in vitro* models.

Cell/Cell line	PC concentration	Oxidant challenge	Reference
RAW 264.7	50–250 $\mu\text{g}/\text{mL}$ for 24 h	Lipopolysaccharide (1 $\mu\text{g}/\text{mL}$) for 24 h	Cherng et al. (2007)
Erythrocytes	10–100 nM extract for 13 min	Lipid peroxidation 30 mM AAPH for 4–6 h	Benedetti et al. (2004)
Platelets	0.5–10 nM 3 min before oxidant challenge	Platelet aggregation	Hsiao et al. (2005)
Mononuclear cells	3 μM pre-treatment for 4 h and co-incubation for 2 h	Irradiation 2 Gy	Ivanova et al. (2010)
Cardiomyocytes	10 μM for 1 h and subsequent co-incubation with DOX for 24 and 48 h	DOX 1 μM for 24 and 48 h	Khan et al. (2008a)
LO2	31–250 $\mu\text{g}/\text{mL}$ post treatment for 48 h	CCl_4 (10 mmol/L) for 6 h	Ou et al. (2010)
BV-2	89–150 $\mu\text{g}/\text{mL}$ co-treatment for 24 h	Lipopolysaccharide (1 $\mu\text{g}/\text{mL}$) for 24 h	Chen et al. (2012)
Cerebellar granule cells	1–3 mg/mL pre-treatment for 24 h and co-treatment for 24 h	Potassium and serum deprivation for 24 h	Rimbau et al. (2001)
MDCK	5–50 mM pre-treatment for 1 h and then co-treatment	Oxalate (0.1 mM) for 24 h	Farooq et al. (2014)

DOX: doxorubicin, AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride, CCl_4 : carbon tetrachloride.

as a complementary agent to the traditional anticancer drugs, designed to limit the growth of cancer cells, and for reducing the toxic side effects of these drugs. The knowledge of PC photodynamic properties, mainly its binding capacity to cancer cells, has suggested the use of this protein in the colocalization of tumors *in vivo* (Morcos, Berns, & Henry, 1968; Thangam et al., 2013).

6.2. Macrophage cells

In the mouse leukemic monocyte macrophage RAW 264.7 cells, PC inhibited the inflammation induced with lipopolysaccharide by the inhibition of NO production and of the inhibitor of κB ($\text{I}\kappa\text{B}-\beta$). Also it inhibited the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) activation and

In vitro studies

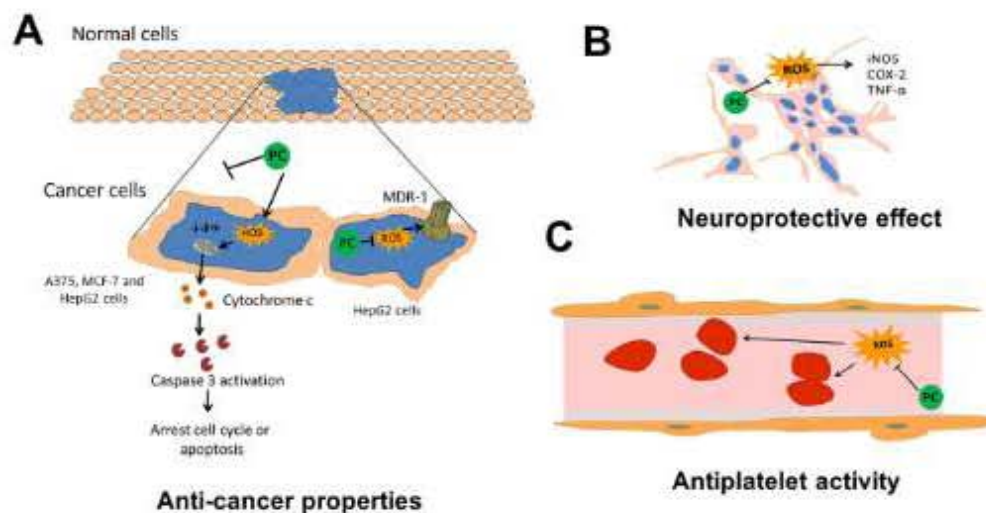


Fig. 5 – *In vitro* properties of PC. (A) Anticancer properties of PC. This protein induces apoptosis by intrinsic pathway characterized by a reduction in the mitochondrial membrane potential, the release of cytochrome c by increased reactive oxygen species (ROS). On other hand, PC prevents the multidrug resistance protein (MDR-1) formation, and consequently, cancer resistance. (B) Neuroprotective effect in microglial BV-2 cells. In these cells PC reduces the lipopolysaccharide-induced expression of inducible nitric oxidativase synthase (iNOS), cyclooxygenase-2 (COX-2) and TNF- α . (C) antiplatelet properties. PC is a potent inhibitor of agonist-induced human platelet aggregation because it prevents the effects of ROS as second messengers in the initial phase of platelets activation.

tumor necrosis factor- α (TNF- α) formation (Cheng et al., 2007, Fig. 5).

6.3. Blood cells

PC extracts attenuated the 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced hemolysis, lipid peroxidation and oxidation of glutathione (GSH) in erythrocytes and plasma, from humans and rats (Benedetti et al., 2004).

In the field of hemostasis, Hsiao et al. (2005) showed that PC is a potent inhibitor of agonist-induced human platelet aggregation because it prevents the effects of ROS as second messengers in the initial phase of platelets activation (Fig. 5C). According to these results, PC may be a useful agent in the antiplatelet therapy in patients diagnosed with arterial thrombosis (Hsiao et al., 2005).

When human lymphocytes are irradiated with 2 Gy (^{137}Cs gamma source, dose rate 1.92 Gy/min) a reduction on antioxidant enzymes was observed. Nevertheless, the pre-treatment with PC for 2 h prevented the reduction in the levels of catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferase (GST) (Ivanova et al., 2010) (Table 3).

6.4. Neuronal cells

The knowledge of agents that decrease the undesirable effects on the brain represents one of the main research areas worldwide. PC has shown beneficial effects in neuronal cells. For example, it acts as a neuroprotective agent (prevention for 24 h) against apoptosis induced by potassium and serum (K/S) removal. These effects were achieved by inhibiting ROS induced by K/S deprivation in cerebellar granule cells (Rimbau et al., 2001).

On the other hand, PC has shown to induce favorable effects in human neuroblastoma SH-SY5Y cells because it attenuates, in a dose-dependent manner, tert-butyl hydroperoxide-induced cell death. This effect is explained, at least in part, by its antioxidant activity and iron chelating which stop lipid peroxidation and damage (Bermejo et al., 2008; Marin-Frida et al., 2012). Microglial BV-2 cells are another example where PC decreased the lipopolysaccharide-induced mRNA levels for inducible nitric oxidative synthase (iNOS), COX-2, TNF- α and interleukin 6 (IL-6) (Chen et al., 2012, Fig. 5B). These findings suggest the use of PC as a preventive agent in neurodegenerative disorders where oxidative stress is involved. Nevertheless, it is necessary to design clinical studies to know whether PC may or may not represent an alternative for the treatment of neurological diseases.

6.5. Thymocytes

Tributyltin is a member of the organotin family; it is extensively used as a biocide. However, this agent is associated with thymus damage and oxidative stress. In isolated thymocytes, PC decreased tributyltin induced oxidative damage and apoptosis (Gupta et al., 2011). PC increased GSH levels, and prevented the loss of mitochondrial membrane potential, activation of caspase-3 activity, induction of Bax (an apoptosis promoter) and increased the Bax/Bcl-2 ratio (Gupta et al., 2011). Therefore, it was postulated that the beneficial effects of PC were related to both its antioxidant activity and its iron chelating

activity since iron is the mediator of tributyltin-induced damage (Gupta et al., 2011; Suresh et al., 2009).

6.6. Liver cells

PC has shown beneficial effects in liver, kidney and heart cell lines. PC showed protective effects in immortalized normal hepatocyte cell line LO2 treated with carbon tetrachloride (CCl₄) and PC for 6 h (Table 3). Briefly, PC prevented, in a dose-dependent manner, the following alterations induced by CCl₄: cell viability lost, disruption of nuclear envelope and cytoplasm, alanine transaminase (ALT) leakage, ROS production, decrease in the activity of SOD and of GSH levels and in secondary oxidation products represented by malondialdehyde (MDA) levels (Ou, Zheng, Lin, Jiang, & Yang, 2010).

6.7. Renal cells

The pre-treatment of Madin-Darby canine kidney (MDCK) cells with PC for 1 h prevented the reduction of the mitochondrial membrane potential, the increased ROS production, lipid peroxidation and reduced ATP synthesis induced by oxalate (Farooq et al., 2014) (Table 3).

6.8. Cardiac cells

DOX induces cardiac damage associated to the increased ROS production. Primary cultures of cardiomyocytes were pre-treated with PC and subsequently with DOX for 24 h (Table 3). As a consequence, ROS production was attenuated. In addition, PC prevented apoptotic cell death, reduced Bax and increased Bcl-2 levels (an apoptotic inhibitor) and prevented cytochrome c release and caspase-3 activation (Khan et al., 2006b).

6.9. PC crosses through the cell membrane?

The location of PC inside cells is controversial. Using confocal laser fluorescence, PC was localized inside HepG2 cells when these latter were exposed 24 h to a PC concentration between 1 and 100 mM (Nishanth et al., 2010). Conversely, when immunofluorescence was used PC was located into cytosol of K562 cells, but it could not be localized inside cell nucleus after 48 h of treatment with PC. It is important to highlight that K562 cells were treated with saponin to permeabilize the cell membrane allowing the uptake of polyclonal antibodies against PC (Subhashini et al., 2004). Wang et al. (2012) used a similar method to immunolocalize PC inside HepG2 mitochondria (Wang et al., 2012). Furthermore, Wu, Lin, Yang, Weng, and Tsai (2011) used polyclonal rabbit antibodies to immunolocalize PC (0.1 mg/mL) inside murine metastatic melanoma (B16F10) cells. Ten minutes after the treatment, PC was found inside cells, and after 30 min the protein was localized inside nuclei and some minutes later in the cytoplasm (Wu et al., 2011). Another study took advantage of the PC fluorescence characteristics (excitation: 384 nm; emission: 640 nm) to determine whether this protein entered to thymocytes after 24 and 48 h of incubation (Gupta et al., 2011). However, results were not clear to establish whether the fluorescence observed was due to the interaction between PC and microplate or between PC and cells.

It is unknown whether PC requires a transport protein carrier to enter cells. Further studies should be performed to explore this area in the future.

7. In vivo models

Most research related to PC has been conducted on *in vivo* models, mainly in rats and mice. Since the antioxidant activity of PC was determined by *in vitro* assays, this protein has been employed in several animal models where the oxidative stress plays an important role (Table 4 and Fig. 6).

7.1. Anti-inflammatory

Inflammation is a physiological complex process that provides to the organism a response to tissue damage caused by mechanical, chemical or microbial action. ROS can initiate and also perpetuate inflammatory cascades and induce subsequent tissue damage (Kaplan et al., 2007). In this way, antioxidants may be useful to attenuate inflammation. PC was first evaluated in a damage model induced by glucose oxidase (GO). The treatment with PC 1 h before GO displayed anti-inflammatory properties (Romay et al., 1998a) (Table 4). GO reacts with endogenous glucose generating H_2O_2 and $^{\bullet}OH$. Therefore the PC scavenging activity for these ROS was important in this protective effect (Romay et al., 1998a). PC was subsequently employed in other inflammatory models, including ear edema-induced by either arachidonic acid or 12-O-tetradecanoylphorbol-13-acetate (TPA); carrageenan-induced paw edema and cotton pellet granuloma (Romay et al., 1998b). In these models the activation of cyclooxygenase (COX 1 and 2, Fig. 6A), lipoxygenase (LPO), protein kinase C and ROS production were important damage mediators. PC inhibited in a dose-dependent manner the inflammation in all models; however for the TPA model the inhibition was weak (Romay et al., 1998b).

7.2. Liver

The liver is the main organ where exogenous chemicals are metabolized and eventually excreted. As a consequence, liver is exposed to high concentrations of these chemicals, which may result in liver dysfunction, cell injury, and even organ failure (Klaassen, 2007). One of these toxic agents is CCl_4 , which is metabolized by cytochrome P450 to trichloromethyl radical ($\bullet CCl_3$) to initiate lipid peroxidation. Interestingly, PC prevented the CCl_4 -induced hepatotoxicity (Fig. 6B, Table 4). A first study showed that the antioxidant activity of PC prevents the damage induced by free radical formation secondary to the metabolism of CCl_4 by cytochrome P-450 (Vadhiraja et al., 1998). Then, it was shown that PC prevents in a dose-dependent manner the CCl_4 -induced liver damage which was evident by the attenuation in the serum activities of aspartate transaminase (AST) and ALT (Ou et al., 2010). PC prevents the oxidative damage by measuring MDA and GSH level, and the activity of antioxidant enzymes SOD and glutathione peroxidase (GPx). In addition, PC significantly decreased the mRNA levels of transforming growth factor-beta1 (TGF- β 1) and hepatocyte growth

factor (HGF). Histological analysis revealed that PC prevents vacuole formation, inflammatory infiltration, edema and necrosis of hepatocytes (Ou et al., 2010).

Another study showed that PC prevents galactosamine-induced hepatic necrosis, leukocyte infiltration and serum increase of AST and ALT activities, as well as of hepatic levels of secondary oxidation products represented by MDA (Gonzalez et al., 2003).

PC protects against hepatic encephalopathy when rats are treated with thioacetamide (AAT) (Sathyasaikumar et al., 2007). In this model, PC increased the survival rate and reduced alterations in ammonia (serum, liver and brain) and serum levels of ALT, AST and albumin. Furthermore, PC improved the prothrombin time. The histological analysis revealed a reduction in necrosis and infiltration of neutrophils and mononuclear cells compared with AAT group. PC attenuates the oxidative damage and the loss of CAT activity in brain. Besides, the transmission electron microscopy showed a reduction of cellular and mitochondrial edema (Sathyasaikumar et al., 2007).

Finally, PC prevented in a dose-dependent manner, the effects of alloxan-induced increase in triglycerides and total cholesterol levels and the expression of glucokinase (GK) in liver homogenate (Ou et al., 2012).

7.3. Heart

The protective effect of PC has also been studied in the field of atherogenesis. Riss et al. (2007) fed hamsters with an atherogenic diet (without vitamins C and E and selenium) with or without a daily dose of PC and PC-Se for 84 days (Table 4). Results showed that PC reduced aortic $O_2^{\bullet-}$ production, expression of p22^{phox} subunit of NADPH oxidase and total cholesterol content, and increased the plasma scavenging capacity in comparison with the non-treated group. Also PC-Se reduced high-density lipoprotein cholesterol (HDL-C) concentrations (Riss et al., 2007).

On the other hand, PC attenuates heart ischemia reperfusion (IR)-induced damage by decreasing ROS formation, apoptotic death, Bax/Bcl-2 ratio, caspase-3 activity, and increase the extracellular signal-regulated kinase (ERK) 1/2 phosphorylation and mitogen-activated protein kinase (p38 MAPK) activation compared with the control group (Khan et al., 2006a).

7.4. Lung

The lung is the main organ affected by paraquat (PQ) that induces the formation of free radicals associated with lipid peroxidation and inflammation (Blanco-Ayala, Andérica-Romero, & Pedraza-Chaverri, 2014). In this regard, PC has shown to attenuate lung damage; it reduces NF- κ B, TNF- α , iNOS and secondary oxidation MDA formation, and increases the activity of SOD and GPx compared to the non-exposed group (Sun et al., 2011) (Fig. 6C, Table 4).

The acute lung injury induced by lipopolysaccharide inhalation was prevented by PC treatment (Leung, Lee, King, Tsai, & Chou, 2013). Such protection was associated to the attenuation of apoptosis (evaluated by the expression of Bax, Bcl-2 and caspase-3), $O_2^{\bullet-}$ production and inflammation that was evaluated by TNF- α , interleukin-1 beta (IL-1 β), IL-6,

Table 4 – Nutraceutical properties of the antioxidant PC on *in vivo* models.

Property	PC dose	Oxidant challenge	Animal species	Reference
Inflammation protection	50–200 mg/kg oral 1 h before	Glucose oxidase (50 U)	Male OF ₁ mice	Romay et al. (1998a)
	50–200 mg/kg ^a	Carrageenan 10 mg/ml (1%)	Male Sprague–Dawley rat	Romay et al. (1998b)
	50–200 mg/kg ^a	AA topic application (25 µg/µL)	Male OF ₁ mice	
	100–300 mg/kg ^a	TPA 4 µg/ear topical application	Male OF ₁ mice	
Hepatoprotection	50–200 mg/kg treatment for 7 days	Cotton pellet 50 mg	Male Sprague–Dawley rat	
	Pretreated with 200 mg/kg i.p. for 1 or 3 h	CCl ₄ (0.6 mL/kg, i.p.)	Male albino rat	Vadraja et al. (1998)
	50, 100 and 200 mg/kg i.p. 1 h before the toxic	R-(+)-pulegone (250 mg/kg, i.p.)	Female Wistar rats	Gonzalez et al. (2003)
	50–200 mg/kg, i.p. 1 h before	Galactosamine (800 mg/kg, i.p.)	Female Wistar Rat	Gonzalez et al. (2003)
	50–200 mg/kg, i.p. 1 h before	Galactosamine (800 mg/kg, i.p.)	Female Wistar Rat	Gonzalez et al. (2003)
Nephroprotection	100–400 mg/kg pre-treatment for 7 days	CCl ₄ (10 mg/ml, i.p. de 0.5% CCl ₄)	Male ICR mice	Ou et al. (2010)
	Co-treatment with 50 mg/kg i.p.	AAT 300 mg/kg/day for 2 days, i.p.	Male Wistar rat	Sathyaakumar et al. (2007)
	300 mg/kg oral for 10 weeks	Diabetic nephropathy	Male C57BL/Ks J db/db mice	Zheng et al. (2013)
	Pre-treatment with 5–30 mg/kg i.p. 1 h before the toxic	Cisplatin 18 mg/kg i.p.	Male CD-1 mice	Fernandez-Rojas et al. (2014)
Multi-organ protection ^b	50–100 mg/kg orally 30 min before the toxic for 5 days	HgCl ₂ 5 mg/kg, i.p.	Male NIH mice	Rodriguez-Sanchez et al. (2012)
	100 mg/kg	Sodium oxalate (70 mg/kg, i.p.)	Male Wistar rats	Farooq et al. (2000)
	Pre-treatment with 4 mg/kg i.p. for three consecutive times	At 7 days after PC was treated with HgCl ₂ 5 mg/kg i.p.	Male Swiss-albino mice	El-Agouty et al. (2007)
	100 and 200 mg/kg, oral daily for 6 weeks	Alloxan 150 mg/kg i.p. at the end of the second week	Male ICR mice	Ou et al. (2012)
Neuroprotection	25–75 mg/kg i.p. for 3, 6 and 12 h after surgery	Global cerebral ischemia-reperfusion	Male Mongolian gerbils	Fentón-Rol et al. (2011a)
	200 mg/kg oral daily for 7 days before surgery			
Brain mitochondria protection	25 mg/kg i.p. for 12 days (therapeutic and prophylactic treatment)	Encephalogen (spinal cord) and <i>Bordetella pertussis</i> (200 × 10 ⁶ organisms/ml)	Male Lewis rat	Fentón-Rol et al. (2011b)
	Pre-treatment with 3–15 30 µM for 5 min	100 µM Ca ²⁺ / 3 mM Pi	Male Wistar rat	Marín-Frutos et al. (2012)
Lung protection	Immediately treated with 50 mg/kg i.p. after paraquat	Paraquat 50 mg/kg orally	Male Wistar rat	Sun et al. (2011)
Cardioprotection	50 mg/kg i.p. 3 h after instillation of LPS	LPS (<i>Escherichia coli</i> 055:B3, 5 mg/kg body weight, i.t.)	Male Sprague–Dawley rats	Leung et al. (2013)
	Perfusion for 15 min with 10 µM before ischemia and throughout reperfusion	Ischemia-reperfusion	Sprague–Dawley rat	Khan et al. (2006a)
Oxidative stress protection	36.3 mg/kg day orally for 12 weeks	Atherogenic diet for 12 weeks without Se, vitamins C and E	Male golden Syrian hamsters	Riss et al. (2007)
Eyes protection	Immediately treated with 200 mg/kg i.p.	Sodium selenite 19 µmol/kg, subcutaneously	Male and female of 9 days old Wistar rat pups	Kumari & Anbarasu, (2014)

AA: arachidonic acid, TPA: 12-O-tetradecanoylphorbol-13-acetate, CCl₄: carbon tetrachloride, AAT: thiocetamide, HgCl₂: mercuric chloride, LPS: lipopolysaccharide, Pi: inorganic phosphorus.

^a Oral route 1 h before.

^b Liver, kidney and pancreas.

cytokine-induced neutrophil chemoattractant 3 (CINC-3) and NF-κB expression. Histological analysis showed that PC reduced edema, thickening of the alveolar walls and neutrophil infiltration (Leung et al., 2013).

7.5. Brain

Brain-IR brings severe sequelae. Although the mechanism of ischemic neuronal death is not fully understood, it is known that

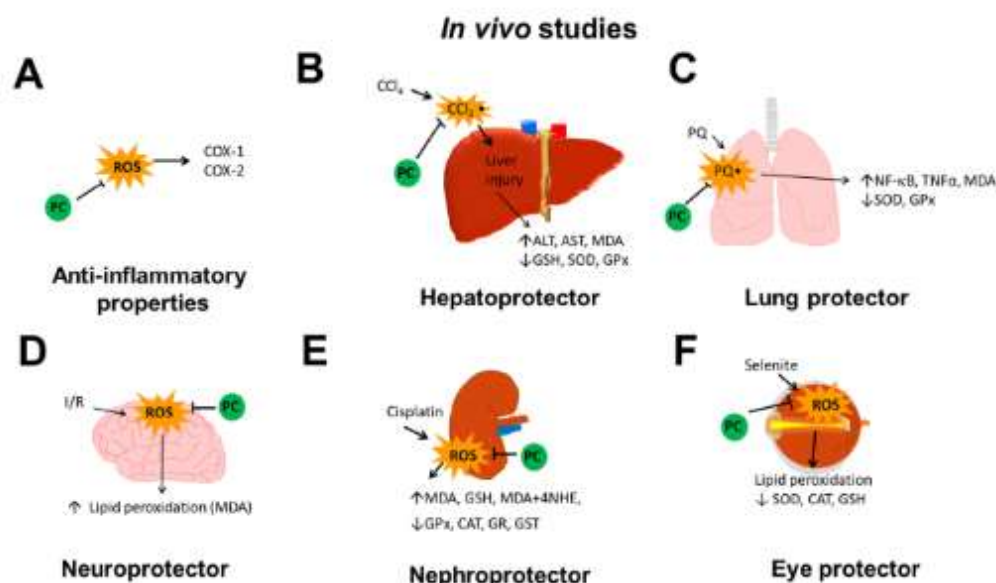


Fig. 6 – Nutraceutical properties of phycocyanin (PC) in vivo studies. (A) In different animal models PC prevents inflammation due to its scavenging activity and by the inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). (B) The CCl₄ liver metabolism mediated by cytochrome P450 produces ROS. The scavenging activity of PC prevents ROS production and liver damage. At the liver, the carbon tetrachloride (CCl₄) is bio-transformed to trichloromethyl radical (\cdot CCl₃). These latter species induce lipid peroxidation, which is counteracted by PC. Also, PC reduces the CCl₄-induced increase of serum aspartate transaminase (AST), alanine transaminase (ALT) and malondialdehyde (MDA) levels, and prevents both CCl₄-induced decrease in glutathione (GSH) levels and in the activity of SOD and glutathione peroxidase (GPx). (C) The redox cycle of paraquat (PQ) produces radical PQ (PQ \cdot). This radical can react with oxygen, leading to the formation of ROS and causing lipid peroxidation. PC attenuates the PQ-induced lung damage by reducing ROS production. Besides, PC reduces MDA levels, kappa-light-chain-enhancer of activated B cells (NF- κ B) activation, tumor necrosis factor- α (TNF- α) expression and formation of inhibitor of κ B (I κ B). Also it prevents the reduction of SOD and GPx activities. (D) ROS generation increases during ischemia/reperfusion (I/R) leading to oxidative damage. PC prevents the oxidative damage induced during I/R, reducing lipid peroxidation evaluated by MDA and peroxidation potential. (E) Cisplatin (CP) is a potent chemotherapeutic agent that induces nephrotoxicity. ROS production is involved in CP-induced nephrotoxicity. PC prevents the CP-induced decreased of antioxidant enzymes such as GPx, catalase (CAT), glutathione reductase (GR), glutathione-S-transferase (GST) and the increase in markers of oxidative damage as MDA, 4-hydroxy-2-nonenal (4-HNE) and GSH content. (F) PC prevents the cataract induced by selenite. PC prevents lipid peroxidation and reduction in antioxidant enzyme activities (SOD, CAT) and GSH content.

oxidative stress is involved. Different doses of PC were used in two different treatments (pre- and post-treatment) using two different administration routes (oral and i.p.) (Table 4). Encouraging results were found. PC increased the survival rate and reduced the infarct area and neurological defects such as posture and gait. Similar results were obtained in the pre-treatment (200 mg/kg, orally) and post-treatment (75–100 mg/kg i.p.). These results indicate that the oral dose of 200 mg/kg is similar to the i.p. injection of 75–100 mg/kg. The prevention model significantly reduced the serum and renal MDA concentration, peroxidation potential, and the ability to reduce ferric ion. In fact, it was proved that 75 mg/kg i.p. (purity = 30%) has similar result than the dose of 25 mg/kg i.p. (purity = 90%). This indicates that PC with low purity (30%) produces very fa-

vorable results and suggests that it is the responsible compound of the beneficial effects found (Pentón-Rial et al., 2011a).

An interesting and unconventional research about mitochondrial from rat brain was reported by Marín-Prida et al. (2012) (Table 4). The use of a micromolar range of PC reduced the Ca²⁺ overload and prevented mitochondrial pore opening, mitochondria swelling, ROS increase and the release of cytochrome c in a dose-dependent manner. Therefore, these results evinced another mechanism of PC protection (Marín-Prida et al., 2012).

Encephalomyelitis (EAE) is a neurological complication caused by viral infections most often related to the respiratory tract. Pre-treatment and post-treatment with PC induce protection against the EAE-induced increase of MDA, oxidized proteins, peroxidation potential and ferric reducing ability

evaluated in plasma and in brain homogenates. However, only pre-treatment with PC prevented the development of EAE, while the post-treatment only reduced clinical signs of the disease probably induced by PC remyelination. Authors hypothesized that PC reach to the parenchyma in a required concentration is needed to influence microglia functions (Pentón-Rol et al., 2011b). In fact, Rimbau, Camins, Romay, González, and Pallàs (1999) showed that PC crosses the blood-brain barrier probably by its bilirubin mimetic function (Rimbau et al., 1999).

7.6. Kidney

The kidney damage induced by calcium oxalate crystals may progress to the formation of kidney stones (Farooq, Asokan, Sakthivel, Kalaiselvi, & Varalakshmi, 2004). This phenomenon implicates the induction of ROS, lipid peroxidation and decreased GSH content. As in other organs, the use of PC prevents these undesirable effects (Farooq et al., 2008) (Table 4).

On the other hand, the pre- and post-treatment with PC prevented the mercuric chloride (HgCl₂)-induced kidney damage in mice. To achieve these results, three repeated doses of PC were used to prevent the increase of blood urea nitrogen (BUN), creatinine and the red cell count. The authors concluded that one of the possible mechanisms involved was the inhibition of lipid peroxidation. However, lipid peroxidation was never measured (El-Ayouty, Baredy, & Salama, 2007). In contrast, another study revealed that PC prevents lipid peroxidation. In addition, PC prevented the ROS production and the reduction of glutathione disulphide (GSSG) in mice kidneys treated with HgCl₂. The histological analysis showed that PC prevented cellular damage, edema, cellular atrophy of distal and proximal tubules and nucleus loss. These beneficial effects were achieved probably due to the antioxidant activity and the chelating property of PC for removal of mercury (Rodríguez-Sánchez, Ortiz-Buonafina, Blas-Valdivia, Hernández-García, & Cano-Europa, 2012).

In another animal model, the pre-treatment with PC prevented the increase of MDA and total antioxidant capacity in kidney homogenate of mice treated with alloxan (Ou et al., 2012).

Furthermore, PC prevented the cisplatin-induced renal damage. One of the pathological mechanisms of cisplatin in the kidney is the oxidative damage, therefore the use of antioxidants may be a strategy to counteract the cisplatin-induced alterations, and perhaps it may contribute to reduce the prevalence of some kidney diseases. A single dose of PC prevented the renal damage (creatinine, BUN), the reduction of antioxidant enzymes such as GPx, CAT, glutathione reductase (GR), GST, and the increase in markers of oxidative damage as MDA and 4-hydroxy-2-nonenal (4-HNE) and GSH reduction (Fernández-Rojas et al., 2014). Evidences suggest that the antioxidant activity of PC is involved against the cisplatin-induced ROS production (Figure 6E, Table 4).

As mentioned before, PC is composed of an apoprotein and PCB with similar structure to biliverdin. In fact, it is believed that PC mimetics the biliverdin function (Fernández-Rojas et al., 2014; Marin-Frida et al., 2013; McCarty, 2011; Zheng et al., 2013). A study tested PC and PCB in db/db and db/+ mice. PC prevented albuminuria and mesangial expansion and increased MDA levels, NADPH oxidase

expression, 8-hydroxy-2'-deoxyguanosine (8-OHdG), tumor growth factor- β and fibronectin expression. Interestingly, a dose of PC 300 mg/kg given orally for 10 weeks showed the same protective effect than the oral administration of PCB 15 mg/kg for 2 weeks (Zheng et al., 2013). Therefore, this finding indicates that PCB may represent the main compound of PC that shows this beneficial effect and probably, it is also involved with other beneficial properties.

7.7. Eyes

A cataract is a clouding of lens inside the eye that decreases the vision; its development is often a gradual process of normal aging. Nevertheless, Taylor (1999) has described other factors: daylight, diet, diabetes, dehydration, among others. The latter catch-all category probably involves genetic influences in nuclear and cortical opacification in humans. The final common pathway by which these factors exert their influence is predominantly due to oxidation of lens proteins and peroxidation of lipids (Kothadia et al., 2011). In this regard, the cataract model induced with selenite was employed in Wistar rats pups of 9 day old (Kumari & Anbarasu, 2014). Rats that received PC (200 mg/kg i.p.) and selenite at same time prevented the cataract process and the subsequent damage to the eye lens that was associated to the prevention of the activity of SOD and CAT and GSH content (Kumari & Anbarasu, 2014) (Figure 6F, Table 4).

7.8. Pancreas

Alloxan is a toxic compound analogous to glucose that destroys pancreatic beta cells inducing type 1 diabetes mellitus. In a recent study, some mice were exposed to alloxan and were treated with PC. As a consequence, PC counteracted the glycosylated proteins and glucose serum levels. Also, PC reduced the MDA content and attenuated the loss of total antioxidant capacity in a dose-dependent manner. In addition, PC reduced the p53 expression. Furthermore, the histological examination revealed that PC re-sets the number of β cells of insulin-secreting glands. Thus, the use of PC promoted the regeneration of pancreatic alloxan-induced damage beta cells (Ou et al., 2012).

8. Final remarks

Over the years, it has been shown that PC acts as a nutraceutical compound as described in this contribution. The pathophysiology of many diseases comprises the imbalance between oxidant species and those with antioxidant activity causing oxidative damage. In this way, the evidence supports the benefits of nutraceuticals in the treatment and prevention of diseases. In fact, some researchers have suggested using nutraceuticals as adjuvants in the clinical practice (Brithwaite et al., 2014; Ferguson, 2009). Among nutraceuticals, vitamin D is related to a reduction on atherosclerosis, insulin resistance, dyslipidemia and hypertension (Menezes, Lamb, Lavie, & DiNicolantonio, 2014). A dietary supplemental containing gamma linolenic acid, vitamin E, vitamin C, beta carotene and coenzyme Q10 may be considered in the treatment and prevention of dry skin associated with the use of oral isotretinoin

(Fabbrocini et al., 2014). The administration of omega-3 can effectively improve endothelial function in adolescents with metabolic syndrome by reducing serum levels of vascular endothelial growth factor (VEGF), as a major index for atherosclerosis progression and endothelial destabilization (Alumadi et al., 2014). The Mediterranean style diet improves the glucose and lipid profile in children and adolescents with obesity and metabolic syndrome (Velazquez-López et al., 2014). The co-administration of curcumin capsules with glyburide may be beneficial to improve glycemic control in patients. The lipid lowering and antidiabetic properties of the curcumin show this antioxidant as a potential future drug molecule (Neerati, Devde, & Gangi, 2014). The use of PC has been shown to prevent the imbalance "antioxidants/oxidants" at least in *in vitro* and *in vivo* models (See Table 2, 3 and 4); however further studies should be performed to know whether this nutraceutical might be used in the clinical practice. Therefore, it is essential to determine the LD₅₀, the metabolites produced during PC metabolism when is administrated by different routes (oral and i.p.), determine whether whole PC or its derivatives are responsible of its biological effects. Besides, the mechanism by which PC crosses the plasma membrane has to be established. As mentioned in this review, PC has antioxidant effects in more than one organ and in different disease models (summarized in Fig. 6 and Table 4). Therefore, the knowledge of PC properties should be used by researchers to develop future studies and clinical trials to decrease the prevalence of human diseases.

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13.2 C-phycoerythrin prevents cisplatin-induced nephrotoxicity through inhibition of oxidative stress

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C-Phycocyanin prevents cisplatin-induced nephrotoxicity through inhibition of oxidative stress

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The aim of this study was to evaluate whether the antioxidant C-phycoerythrin (C-PC, 5–50 mg kg⁻¹ i.p.) was able to prevent cisplatin (CP, 18 mg kg⁻¹ i.p.) induced nephrotoxicity by reducing oxidative stress in CD-1 mice. Nephrotoxicity was assessed by measuring blood urea nitrogen, plasma glutathione peroxidase, plasma creatinine, the renal activity of *N*-acetyl-β-D-glucosaminidase, apoptosis and histopathological changes. Oxidative stress was evaluated by measuring the content of glutathione, malondialdehyde, 4-hydroxynonenal and oxidized proteins in renal tissue. C-PC prevented CP-induced renal damage and oxidative stress in a dose-dependent manner. Moreover, C-PC prevented the decrease in the renal activity of the antioxidant enzymes glutathione peroxidase, glutathione reductase, glutathione-S-transferase and catalase induced by cisplatin. *In vitro* assays showed that C-PC was an effective scavenger of the following reactive species: hypochlorous acid, peroxynitrite anions, peroxy radicals, diphenyl-1-picrylhydrazyl, hydroxyl radicals, superoxide anions, singlet oxygen and hydrogen peroxide. It is concluded that the protective effect of the nutraceutical C-PC against CP-induced nephrotoxicity was associated with the attenuation of oxidative stress and the preservation of the activity of antioxidant enzymes.

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1. Introduction

There is huge interest among consumers and the food industry in products that can promote health and well-being named functional foods (FF).¹ The beneficial action exerted by FF is due to some components called functional ingredients or nutraceuticals. They have been described as “any substance that is a food or part of a food that provides medical and/or health benefits, including the prevention and treatment of disease”.² In this way, C-phycoerythrin (C-PC) is a nutraceutical compound that gives the characteristic blue-green color to the FF *Spirulina maxima*. C-PC is a biliprotein composed of two subunits, α and β proteins, which are bound to the chromogen phycocyanobilin (PCB); one PCB is joined to the α subunit and two PCB are joined to the β subunit.³ PCB is an open-chain tetrapyrrole with a chemical structure similar to biliverdin. It seems to be susceptible to biliverdin reductase, giving rise to phycocyanorubin, an analog of bilirubin.⁴ It is used in the food industry,

cosmetics, biotechnology and medical practice.⁵ Some of its nutraceutical properties are: antioxidant, anti-inflammatory, anti-neoplastic, and anti-diabetic, among others. In fact, its antioxidant activity is involved in the protective effect against liver, neuronal and renal damage.^{6,7} On the other hand, cisplatin [*cis*-diammine-dichloroplatinum(II) (CP)] is a potent chemotherapeutic agent widely used for the treatment of various types of tumors worldwide⁸ playing a major role in the treatment of many types of cancers, such as testicular, bladder, ovarian, head and neck, cervical, lung and colorectal malignant tumors.^{9,10} However, its clinical use is limited by side effects, mainly nephrotoxicity which affects 30% of patients who use it.⁸ Evidence suggests that the formation of reactive oxygen species (ROS), the decrease in the activity of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST), inflammation, mitochondrial dysfunction and apoptosis are involved in CP-induced nephrotoxicity.^{11,12} It has been found previously that C-PC prevents apoptosis in mice with CP-induced nephrotoxicity.¹¹ However, it has never been studied whether the C-PC protection against CP-induced nephrotoxicity is associated with the prevention of oxidative stress and the preservation of antioxidant enzyme activity. Thus, it was decided to study whether the protection by the nutraceutical C-PC against the CP-induced nephrotoxicity is associated with the prevention of oxidative damage and preservation of the activity of antioxidant enzymes.

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In addition the *in vitro* ROS scavenging activity of C-PC used in the present study was also characterized.

2. Results and discussion

2.1 Purity of the C-PC

C-PC is a nutraceutical compound with antioxidant properties. It prevents oxidative damage and is used for human consumption as a colorant in food and milk shakes, and as an

ingredient in cosmetic and pharmaceutical formulations. The α and β subunits were determined to characterize the purity of the C-PC sample used in this work. Fig. 1 shows the characteristic peak absorption of PCB at 620 nm, and the two bands for α and β C-PC subunits. The purity of the C-PC used in this study was 2.5. C-PC from the same source (Parry Nutraceuticals) or from a similar purity has been used in other studies.^{13–15} Also, the spectrum shows the absence of other phycobiliproteins as allophycocyanin (652 nm) and phycoerythrin (562 nm).³

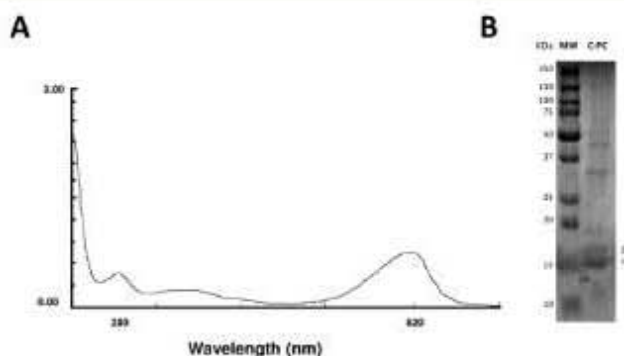


Fig. 1 Evaluation of C-PC purity. (A) Absorption spectra of C-PC (250–700 nm). The characteristic absorption peak of phycocyanobilin (PCB) at 620 nm is observed. (B) SDS-PAGE. The gel shows molecular weight markers (MW, 7 μ L) and the sub-units α and β of C-PC (C-PC: 8 μ g of protein). The ratio A620/A290 is 2.5.

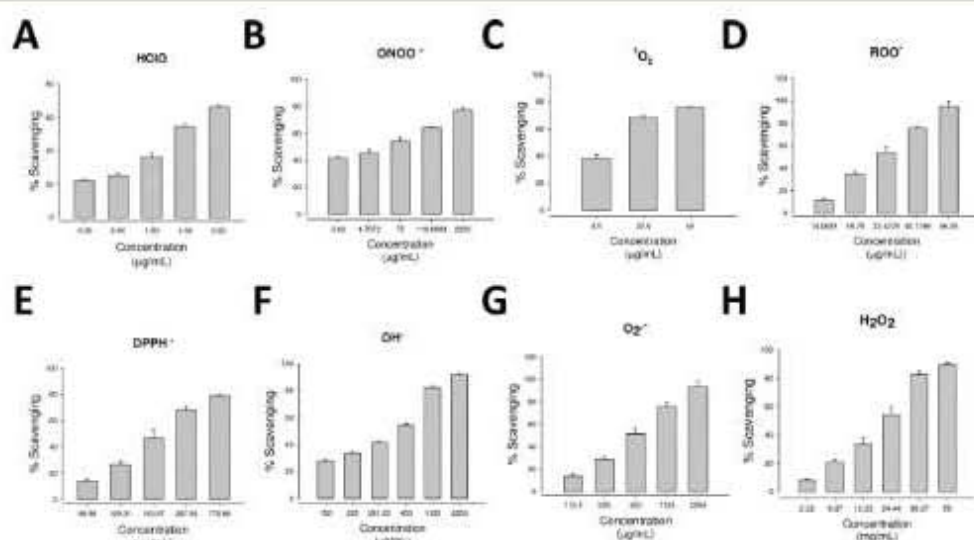


Fig. 2 C-PC scavenging activity: (A) HClO, (B) ONOO⁻, (C) ¹O₂, (D) ROO[·], (E) DPPH[·], (F) OH[·], (G) O₂^{·-}, (H) H₂O₂; HClO, hypochlorous acid; ONOO⁻, peroxynitrite anion; ROO[·], peroxy radical; DPPH[·], 2,2-diphenyl-1-picrylhydrazyl; OH[·], hydroxyl radicals; O₂^{·-}, superoxide anion; ¹O₂, singlet oxygen; H₂O₂, hydrogen peroxide. Data are shown as mean \pm SEM; $n = 3$.

Table 1 Scavenging ability of C-phycoerythrin (C-PC) and reference compounds. Data are expressed as IC₅₀^a (μg mL⁻¹)

Compound	HClO	ONOO ⁻	ROO [•]	DPPH [•]	OH [•]	O ₂ ^{-•}	¹ O ₂	H ₂ O ₂
C-PC	4.9 ± 0.2	5 ± 0.6	20 ± 6	197 ± 7	334 ± 9	450 ± 5	11 670 ± 1	21 758 ± 2302
Lipoic acid	1148 ± 132	—	—	—	—	—	214 ± 85	—
Penicillamine	—	1.01 ± 0.26	—	—	—	—	—	—
Trolox	—	—	1.19 ± 0.03	—	—	—	—	—
Vitamin C	—	—	—	51.15 ± 4.19	—	—	—	—
DMTU	—	—	—	—	2.6 ± 0.62	—	—	—
NDGA	—	—	—	—	—	0.18 ± 0.02	—	—
Pyruvate	—	—	—	—	—	—	—	0.28 ± 0.001

^a HClO, hypochlorous acid; ONOO⁻, peroxynitrite anion; ROO[•], peroxy radical; DPPH[•], diphenyl-1-picrylhydrazyl; OH[•], hydroxyl radical; O₂^{-•}, superoxide anion; ¹O₂, singlet oxygen; H₂O₂, hydrogen peroxide. NDGA, nordihydroguaiaric acid; DMTU, dimethylthiourea. IC₅₀ = ability of the sample to scavenge 50% of each reactive oxygen species. Data are shown as mean ± SEM; n = 3.

2.2 *In vitro* antioxidant activity

First, the *in vitro* ROS scavenging properties of C-PC were characterized. This nutraceutical compound was challenged by several ROS synthesized *in vitro* that were scavenged even up to 90%, such as peroxy radicals (ROO[•]), hydroxyl radicals (OH[•]) and superoxide anions (O₂^{-•}) as shown in Fig. 2. In fact, C-PC scavenges all ROS tested in a concentration-dependent way. The concentration that reduces the ROS tested by 50% (IC₅₀) is summarized in Table 1. C-PC was more efficient at scavenging hypochlorous acid (HClO) than the reference scavenging compound and less efficient at scavenging the other ROS tested.

The decreasing order of scavenging efficiency was: HClO > peroxynitrite anions (ONOO⁻) > ROO[•] > diphenyl-1-picrylhydrazyl radicals (DPPH[•]) > OH[•] > O₂^{-•} > singlet oxygen (¹O₂) > hydrogen peroxide (H₂O₂). Our results are consistent with the ability of C-PC to scavenge HClO, ONOO⁻, ROO[•], DPPH[•], OH[•] and O₂^{-•} reported in previous studies.^{33,37} Nevertheless, this is the first time that the ability of C-PC to scavenge H₂O₂ and ¹O₂ has been reported. Based on the antioxidant properties of C-PC, it was decided to investigate whether C-PC prevents CP-induced nephrotoxicity by reducing oxidative stress markers and by preservation of the activity of antioxidant enzymes in an *in vivo* model in CD-1 mice.

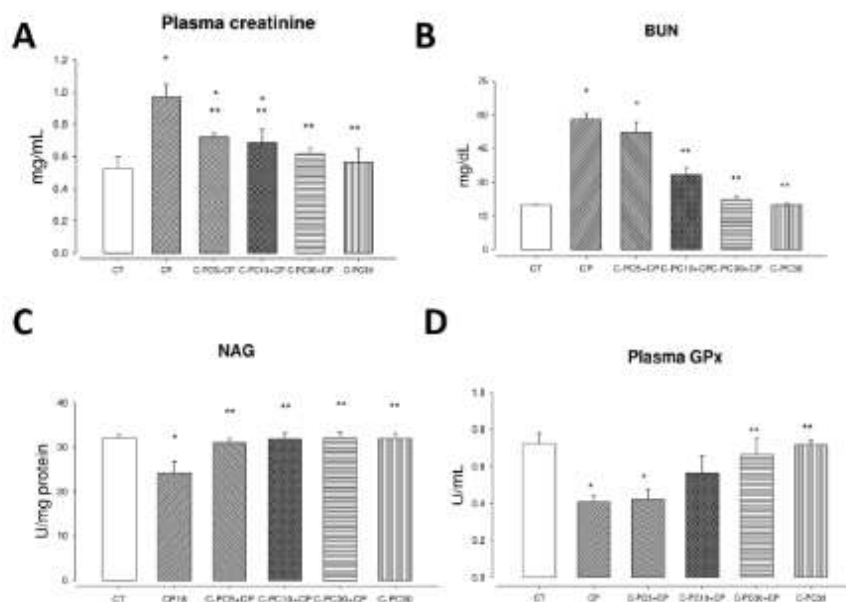


Fig. 3 Protective effect of C-PC against renal damage induced by CP in CD-1 mice. (A) Plasma creatinine, (B) blood urea nitrogen (BUN), (C) N-acetyl-β-D-glucosaminidase (NAG) in renal homogenates, (D) glutathione peroxidase (GPx) in plasma. CT: control, CP: cisplatin 18 mg kg⁻¹ i.p., C-PC 5, 10, 30: phycoerythrin 5, 10, 30 mg kg⁻¹ i.p., respectively. Data are shown as mean ± SEM; n = 5–6. *P < 0.05 vs. CT, **P < 0.05 vs. CP.

2.3 *In vivo* experimental model

2.3.1 Determination of renal function. Nephrotoxicity was characterized by a marked increase in both blood urea nitrogen (BUN) and creatinine in plasma induced by a single dose of CP (18 mg kg⁻¹ i.p.) and was confirmed by the determination of *N*-acetyl- β -D-glucosaminidase (NAG) and plasma GPx and by the histopathological studies. There was an increase (2 to 3-fold) of BUN ($P < 0.05$) and creatinine ($P < 0.001$) in mice treated with CP compared to control mice. Damage was prevented when mice were pre-treated with C-PC at doses of 10 (Fig. 3A, $P < 0.01$) and 30 mg kg⁻¹ (Fig. 3B, $P < 0.05$), but not with 3 mg kg⁻¹. Moreover, NAG is a lysosomal renal enzyme found in proximal tubular cells and its urinary excretion increases when these cells are damaged, thereby, NAG is a specific marker of tubular damage. NAG activity in renal homogenates decreased by 34% in the CP-treated group. Another marker of tubular damage is plasma GPx that is synthesized in renal proximal tubules. The decrease in plasma GPx in the CP-treated group indicates fair damage at the CP target site. Plasma GPx activity was reduced by 43.5% in the CP group compared to the control one (Fig. 3D, $P < 0.05$,

evidencing kidney damage. These alterations were prevented by C-PC in a dose-dependent manner. These parameters were unchanged in the C-PC group (Fig. 3). Our data show that C-PC was able to prevent CP-induced nephrotoxicity which was evaluated by four markers (plasma creatinine, BUN, the renal activity of NAG and plasma GPx).

2.3.2 Histopathological analysis. Light microscopy revealed structural alterations specifically in proximal convoluted tubules in the CP-treated mice, these were characterized by cellular edema, and dead and detached cells (Fig. 4). The automated morphometry analysis showed $52 \pm 5\%$ of proximal tubules with these histological abnormalities, and the surface area of damaged epithelium was of $58 \pm 8\%$ [Fig. 4, $P < 0.05$ vs. control (CT)]. These alterations were prevented by 30 mg kg⁻¹ of C-PC (11%, $P < 0.001$ vs. CP). No histological abnormalities were seen in the C-PC group (30 mg kg⁻¹). C-PC prevented not only renal functional damage (BUN, plasma GPx, plasma creatinine and the renal activity of NAG) but also structural damage (histopathological changes such as necrosis, edema and vacuolization). Pre-treatment of C-PC reduced the above

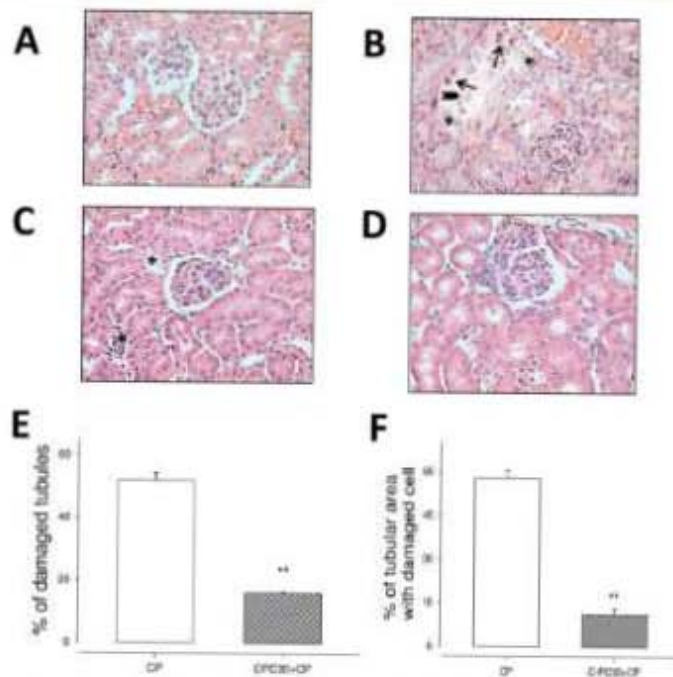


Fig. 4 Representative histology and automated morphometry study. (A) Normal kidney histology in control mouse. (B) Cellular edema (asterisks), dead (arrows) and detached cells (arrow head) of proximal tubular epithelium from mouse treated with CP. (C) In contrast, occasional tubules show damage (asterisks) in a mouse treated with C-PC30 + CP. (D) There are no histological abnormalities in the kidney from mouse treated with 30 mg kg⁻¹ of phycocyanin. (E) Automated morphometry showed a high percentage of damaged convoluted proximal tubules in animals treated with CP than in mice that received C-PC30 + CP. (F) The percentage of the damaged epithelium in injured proximal tubules is higher in CP treated mice than in animals that received C-PC30 + CP. ** $P < 0.05$ vs. CP.

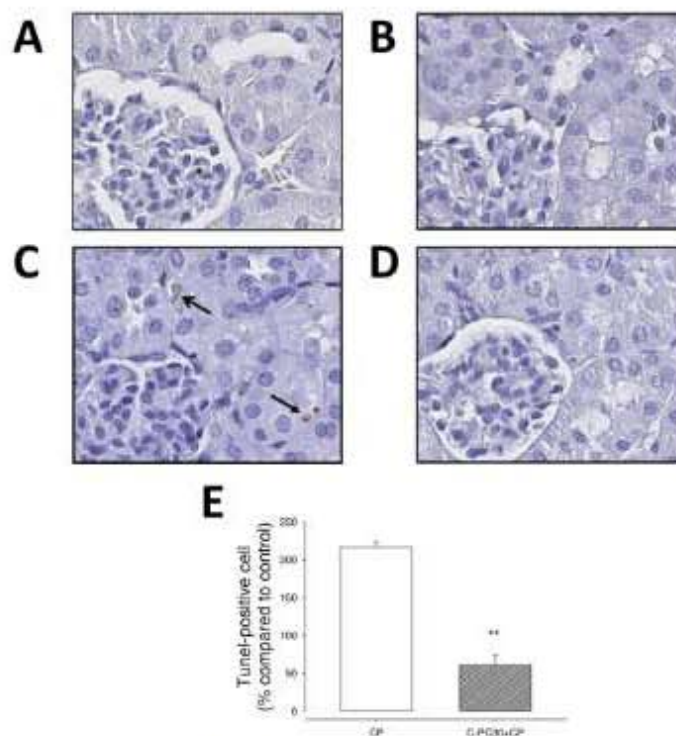


Fig. 5 Immunohistochemical detection of apoptosis by TUNEL (40 \times). (C) Sections of renal cortex of mice treated with CP show intensely stained nuclei (arrows) which indicate tubular cells in apoptosis. (D) Apoptosis was prevented in the C-PC30 group. Basal staining was just observed in renal sections from (A) CT and (B) C-PC30 groups. (E) Quantitative analysis confirms a remarkable protection of C-PC against CP-induced apoptotic death. Data are shown as mean \pm SEM. ** $P < 0.01$ vs. CP.

described renal injury markers in a dose-dependent manner. These data are consistent with the protection of C-PC observed in the functional renal markers described in Section 2.3.1.

2.3.3 Apoptosis detection. Terminal-deoxynucleotidyl-transferase mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL)-positive tubular cells in kidneys of mice treated with CP are shown in Fig. 5. Strongly stained nuclei were observed depicting early apoptosis in some tubules. TUNEL positive cells were clearly decreased by 30 mg kg⁻¹ of C-PC (Fig. 5). Quantitative data, expressed as the percentage of TUNEL-positive cells compared to control, revealed that TUNEL positivity was 2-fold higher in the CP group ($P < 0.05$), while this increase was prevented by 30 mg kg⁻¹ of C-PC ($P < 0.007$ vs. CP, Fig. 5). These results are in accord with those of Lim *et al.*¹² who found that C-PC prevented CP-induced apoptosis.

2.3.4 Oxidative stress markers. The redox status was evaluated by the activity of antioxidant enzymes and by markers of oxidative stress that were altered in the group treated with CP. This group showed a marked decrease of 25% of

glutathione (GSH) content ($P < 0.001$), the main water-soluble antioxidant. Also, an increase in the content of malondialdehyde (MDA) (2.3-fold, $P < 0.001$), 4-hydroxy-2-nonenal (4-HNE) (4.6-fold, $P < 0.05$) and oxidized proteins (1.4-fold, $P < 0.001$, Fig. 6) compared to the control was found. These data agree with previous studies^{18,19} and suggest that oxidative damage is involved in the mechanisms of CP induced nephrotoxicity. These changes were prevented by C-PC in a dose-dependent manner ($P < 0.05$ vs. CP).

2.3.5 Antioxidant enzymes. The activity of the antioxidant enzymes GPx, GST, CAT and GR was decreased in mice from the CP-group by 40, 23, 40 and 11%, respectively ($P < 0.001$ vs. CT, Fig. 7). The protective effect was associated with the prevention of CP-induced alterations in both oxidative stress markers and activity of antioxidant enzymes in a dose-dependent way. GSH is both a ROS scavenger and a cofactor of the antioxidant enzymes GPx and GST. Thus, an imbalance of GSH homeostasis increases ROS levels, leading to oxidative damage. Our results demonstrate that all markers of kidney damage were reduced to values close to baseline by a single dose of C-PC with better

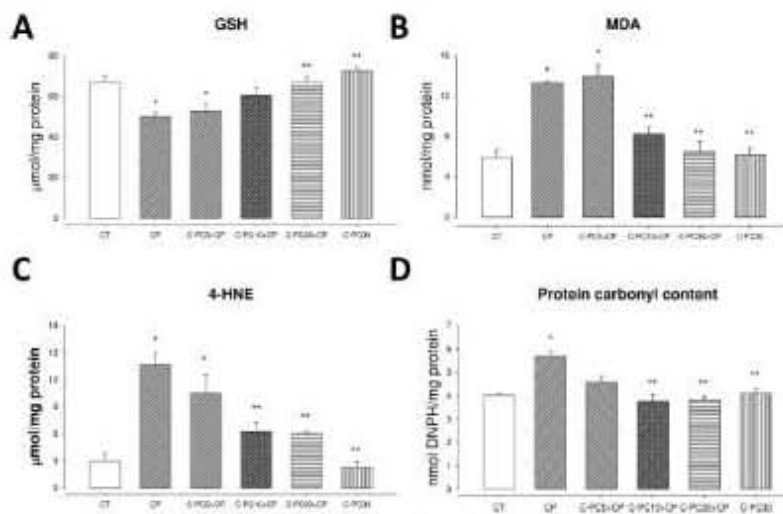


Fig. 6 Oxidative stress markers in renal homogenates. (A) Glutathione (GSH), (B) malondialdehyde (MDA), (C) 4-hydroxy-2-nonenal (4-HNE), and (D) protein carbonyl. CT: control, CP: cisplatin 18 mg kg⁻¹ i.p., C-PC 5, 10, and 30: phycocyanin 5, 10, and 30 mg kg⁻¹ i.p., respectively. Data are shown as mean ± SEM; n = 4–9. *P < 0.05 vs. CT, **P < 0.05 vs. CP.

results at doses of 10 and 30 mg kg⁻¹ 1 hour before CP. Moreover, CP decreased the activity of GPx, GR, GST and CAT. Similar results have been reported by Chakraborty *et al.*,²⁸ Guerrero-

Beltrán *et al.*,¹⁹ and Chirino and Pedraza-Chaverri.²¹ C-PC prevented this decrease in a dose-dependent manner. These data suggest that the renoprotective effect of C-PC is mediated, at

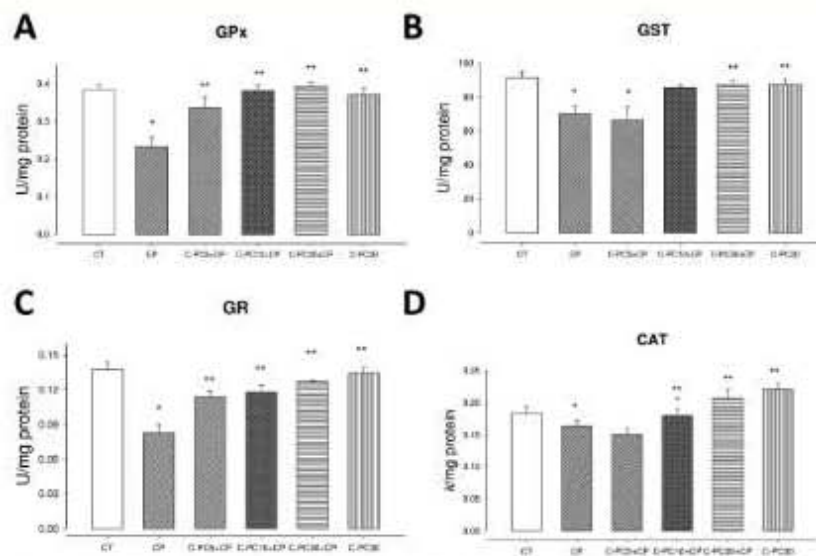


Fig. 7 Activity of antioxidant enzymes in renal homogenates. (A) Glutathione peroxidase (GPx), (B) glutathione-S-transferase (GST), (C) glutathione reductase (GR), and (D) catalase (CAT). CT: control, CP: cisplatin 18 mg kg⁻¹ i.p., C-PC 5, 10, and 30: phycocyanin 5, 10, and 30 mg kg⁻¹ i.p., respectively. Data are shown as mean ± SEM; n = 4–9. *P < 0.05 vs. CT, **P < 0.05 vs. CP.

least in part, by the preservation of the activity of the antioxidant enzymes and support the hypothesis that the mechanism of CP induced nephrotoxicity is related to the depletion of renal antioxidant enzymes.

CP induced renal damage by accumulation in the tubular epithelium, specifically in the S3 segment of the proximal tubule.²¹ It causes an increase in both BUN and creatinine and a decrease in glomerular filtration rate.²² Several mechanisms are involved in the CP-induced nephrotoxicity such as inflammation, mitochondrial dysfunction, necrosis, apoptosis and oxidative stress.^{23–25} Some studies have demonstrated the role of ROS as a major cause of kidney damage induced by CP, the species involved in oxidative and nitrosative damage are: $O_2^{\cdot-}$, H_2O_2 , OH^{\cdot} , $ONOO^{\cdot}$, ROO^{\cdot} and 1O_2 .²¹ It is clearly shown in the present study that all these ROS are scavenged by C-PC (see Table 1 and Fig. 2). These data strongly suggest that the ROS scavenging properties of C-PC are involved in the renoprotective effect against CP-induced damage. Our data are also consistent with the renoprotective effect of the alga *Spirulina platensis* and C-PC in different animal models.^{12,26–28} Besides, C-PC prevents oxidative damage in other organs of different animal models in which oxidative stress is involved. In fact, Ou *et al.*²⁹ demonstrate that C-PC prevents hepatic and pancreatic oxidative damage in mice treated with alloxan, Sun *et al.*³⁰ showed the protective effect of C-PC in oxidative stress in the lung by paraquat, and Pentón-Rol *et al.*⁸ demonstrated the reduction of oxidative damage in the brain of rats with experimental autoimmune encephalomyelitis.

A recent investigation conducted by Lim *et al.*¹² showed that C-PC (50 mg kg⁻¹ i.p.) given one hour before the injection of CP (12 mg kg⁻¹ i.p.) in mice prevents nephrotoxicity by reducing apoptosis. Moreover, Mohan *et al.*³¹ showed that the algae *Spirulina platensis* at a dose of 1000 mg kg⁻¹ bw for 8 days prevents the CP (6 mg kg⁻¹ i.p.) induced nephrotoxicity, oxidative stress and the decrease in the activity of antioxidant enzymes in rats. Nevertheless, this is the first time in which it has been shown that C-PC prevents the CP-induced oxidative damage (evaluated by the content of protein carbonyl, GSH, MDA, and 4-HNE in renal tissue) and decrease of the activity of antioxidant enzymes (GPx, CAT, GR and GST) in kidney homogenates.

Probably, the prevention by C-PC is due to that PCB mimics bilirubin. Both compounds inhibit the $O_2^{\cdot-}$ producing enzyme NADPH oxidase.²⁸ Interestingly, there is recent epidemiological evidence that patients with Gilbert's syndrome (harmless increased bilirubin in up to 3% of the population) are at greatly reduced risk for coronary disease, hypertension, atherosclerosis, and other illnesses.³² However, bilirubin has high cost and therefore the use of C-PC is an attractive alternative.

In summary, the antioxidant effect of C-PC on oxidative stress in the nephrotoxicity induced by CP has been demonstrated for the first time. The protection was associated with the preservation of antioxidant enzyme activity. Further experiments are required to evaluate whether additional mechanisms are involved in the protective effect of C-PC against CP-induced nephrotoxicity in mice.

3. Materials and methods

3.1 Reagents

C-PC was a generous gift from Parry Nutraceuticals (EID Parry (I) Ltd., India). CP (Cat. no. P4394, Lot# MKBG8405V), 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), fluorescein, *nc*-penicillamine, diethylenetriaminepentaacetic acid (DTPA), DPPH[•], terephthalic acid (TA), ascorbic acid, Amplex Red, horseradish peroxidase (HRP), pyruvate, dimethylthiourea (DMTU), nicotinamide adenine dinucleotide reduced form (NADPH), lipoic acid, GSH, 1-chloro-2,4-dinitrobenzene (CDNB) and *p*-nitrophenyl-*N*-acetyl- β -*D*-glucosaminide, tetramethoxypropane, monochlorobimane, buffered formalin, 1-methyl-2-phenylindole, methanesulfonic acid, dimethylsulfoxide (DMSO), 1,3-diphenylisobenzofuran (DPBF), nordihydroguaiaretic acid (NDGA), oxidized glutathione (GSSG), *p*-aminobenzoic acid, sodium dodecyl sulfate (SDS) and polyacrylamide were from Sigma-Aldrich (St. Louis, MO, USA). Dihydroethodamine 123 (DHR-123) was from Cayman Chemical Co. (Ann Arbor, MI, USA). Trolox was from EMD Millipore (Billerica, MA, USA). Ethylenediaminetetraacetic acid, disodium salt (Na₂EDTA), sodium hypochlorite (NaClO) and H₂O₂ were from JT Baker (Xalostoc, Edo, Mexico, Mexico). Commercial kits for the measurement of BUN and plasma creatinine concentration were from Spinreact (Girona, Spain). *In situ* Cell Death Detection Kit, POB (Cat. no. 11 684 817 910) for apoptosis detection was obtained from Roche Applied Science (Mannheim, Germany). 3,3'-Diaminobenzidine (DAB) was from Dako (Carpinteria, CA, USA). All other reagents were of analytical grade and commercially available.

3.2 Purity determination of C-PC

To determine the purity of C-PC, spectrophotometric readings at wavelengths of 280, 620 and 652 nm were taken (250–700 nm) and the A620/A280 ratio was calculated.³³ Besides, SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed according to Pentón-Rol *et al.*⁸ on 15% polyacrylamide gel at 60 V for 3.5 h.

3.3 *In vitro* antioxidant activity

The experiments were performed on C-PC and reference compounds using a Synergy HT multimode microplate reader (Biotek Instruments, Winooski, VT, USA). The data are expressed as IC₅₀ (μ g mL⁻¹). The reference compounds used were: NDGA (for $O_2^{\cdot-}$), DMTU (for OH^{\cdot}), penicillamine (for $ONOO^{\cdot}$), Trolox (for ROO^{\cdot}), lipoic acid (for 1O_2 and HClO), pyruvate (for H₂O₂) and vitamin C (for DPPH[•]).

3.3.1 $O_2^{\cdot-}$ scavenging assay. The scavenging activity of C-PC was determined by evaluating its ability to decrease formazan production from NBT induced by $O_2^{\cdot-}$ generated by the xanthine-xanthine oxidase system. Briefly, 100 μ L of a solution composed of C-PC (0–22.5 μ g mL⁻¹) or 30 mM phosphate buffer pH 7.0 (0% scavenging tube) or NDGA (0–1.0 μ g mL⁻¹) were added to 800 μ L of the reaction mixture (90 μ M xanthine, 16 mM Na₂CO₃, 22.8 μ M NBT and 18 mM phosphate buffer, pH 7.0).

The reaction started by adding 100 μL of xanthine oxidase (168 U L^{-1}). Finally, the optical density at 293 nm and 560 nm was read to measure uric acid and $\text{O}_2^{\cdot-}$, respectively.³⁴

3.3.2 OH \cdot scavenging assay. OH \cdot was generated by the Fenton reaction.³⁵ 20 μL of a solution of C-PC ($10\text{--}22.5 \mu\text{g mL}^{-1}$) or distilled water (0% scavenging) or DMTU ($0\text{--}10 \mu\text{g mL}^{-1}$) were added to 180 μL of the reaction mixture (0.2 mM ascorbic acid, 0.2 mM FeCl_2 , 0.208 mM Na_2EDTA , 1 mM H_2O_2 , 1.4 mM TA, and 20 mM phosphate buffer, pH 7.4). The mixture was incubated for 30 min at room temperature to obtain a fluorescent product that was measured at excitation and emission wavelengths of 326 nm and 432 nm, respectively.

3.3.3 ONOO \cdot scavenging assay. This anion was synthesized according to Cervantes *et al.*³⁶ and its concentration was determined using $\epsilon_{302 \text{ nm}} = 1670 \text{ M}^{-1} \text{ cm}^{-1}$. DHR-123 was used as an indicator of the presence of the anion. In brief, 25 μL of a solution of C-PC ($0\text{--}22.5 \mu\text{g mL}^{-1}$) or 0.1 M phosphate buffer pH 7.4 (0% scavenging tube) or penicillamine ($0\text{--}15 \mu\text{g mL}^{-1}$) were added to 225 μL of the reaction mixture (100 μM DTPA, 14 μM DHR-123, and 50 μM ONOO \cdot). In the absence of an antioxidant, DHR-123 is oxidized to rhodamine-123, a fluorescent compound which is measured at excitation and emission wavelengths of 500 and 536 nm, respectively.

3.3.4 ROO \cdot scavenging assay. The scavenging activity of the sample was determined by the stability of the fluorescence of fluorescein by ROO \cdot . Briefly, a solution of 25 μL of C-PC ($0\text{--}56.3 \mu\text{g mL}^{-1}$) or 75 mM phosphate buffer, pH 7.4 (0% scavenging tube) or Trolox ($0\text{--}2.5 \mu\text{g mL}^{-1}$) was added to the solution composed of 23 nM fluorescein and 19 mM AAPH. Fluorescence was measured at excitation and emission wavelengths of 485 nm and 520 nm, respectively for 1.5 h at 37 $^\circ\text{C}$. At the end of the assay the area under the curve (AUC) was obtained by Gen 5 software (Biotek Instruments).

3.3.5 $^1\text{O}_2$ scavenging assay. $^1\text{O}_2$ was generated from HClO and H_2O_2 as previously described.³⁴ The reaction consisted of 25 μL of C-PC ($0\text{--}50 \mu\text{g mL}^{-1}$) or 45 mM phosphate buffer pH 7 (0% scavenging tube) or lipoic acid ($0\text{--}150 \text{ mg mL}^{-1}$) to 275 μL of the reaction mixture (1 mM H_2O_2 , 30 μM HClO and 1 μM DPBF). $^1\text{O}_2$ causes a reduction in the fluorescence of DPBF that was determined at excitation and emission wavelengths of 410 nm and 455 nm, respectively.

3.3.6 H_2O_2 scavenging assay. The ability of C-PC to scavenge H_2O_2 was conducted using Amplex Red. The assay was performed with 20 μL of C-PC ($2.2\text{--}50 \mu\text{g mL}^{-1}$) or 50 mM phosphate buffer pH 7.4 (0% scavenging tube) or pyruvate ($0\text{--}6.1 \mu\text{g mL}^{-1}$) and 50 μL of 5 μM H_2O_2 and 100 μL of the reaction mixture (0.1 mM Amplex Red, HRP 0.2 U mL^{-1}). Amplex Red is oxidized in the presence of H_2O_2 to produce resorufin, a fluorescent compound that is measured using excitation and emission filters of 530/25 and 590/35, respectively.³⁴

3.3.7 HClO scavenging assay. The ability of C-PC to scavenge HClO was determined using *p*-aminobenzoic acid which reacts with HClO to produce the fluorescent compound 3-chloro-4-aminobenzoic acid.³⁶ Briefly, 30 μL of C-PC ($0\text{--}9 \mu\text{g mL}^{-1}$) or lipoic acid ($0\text{--}16.6 \text{ mg mL}^{-1}$) or buffer (0% scavenging tube) were mixed with 270 μL of the reaction mixture (0.05 mM HClO, 0.1 mM *p*-aminobenzoic acid in 50 mM $\text{KH}_2\text{PO}_4/\text{KOH}$

buffer, pH 7.4) and fluorescence was determined at excitation and emission wavelengths of 280 nm and 340 nm, respectively.

3.3.8 DPPH \cdot scavenging capacity. The activity of C-PC to scavenge DPPH \cdot was performed by the method described by Coballase-Urrutia *et al.*³⁷ Briefly, 10 μL of C-PC ($0\text{--}22.5 \mu\text{g mL}^{-1}$) or vitamin C ($0\text{--}103 \mu\text{g mL}^{-1}$) or distilled water (0% scavenging tube) were mixed with 290 μL of 100 mM DPPH \cdot (in ethanol). After incubation in the dark for 20 minutes the sample was centrifuged at $100 \times g$ for 10 min and the absorbance at 517 nm was measured.

3.4 *In vivo* experimental model

Fifty four CD1 male mice (30–35 g, 8–9 week-old) were fed with standard chow and water *ad libitum*. They were randomly distributed in 6 groups of 4 mice each: the first group received only the vehicle (saline solution) by intraperitoneal (i.p.) route (control, CT), the second group received C-PC 30 mg kg^{-1} i.p., the third one received a single dose of CP (18 mg kg^{-1} i.p.), and the fourth, fifth and sixth groups received different doses of C-PC (5, 10 and 30 mg kg^{-1} i.p., respectively) one hour before CP administration (18 mg kg^{-1} i.p.).³⁸ Seventy two hours after CP administration, mice were anesthetized with pentobarbital (70 mg kg^{-1} , i.p.) and euthanized by exsanguination sectioning the axillary artery. Blood was collected in Eppendorf tubes with heparin. Right kidneys were immediately dissected and frozen by immersion in liquid nitrogen and prepared the homogenate (1 : 10 m/v) in 50 mM $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7 and used for NAG, oxidative stress markers, and antioxidant enzymes was used. Left kidneys were sagittally sectioned and fixed by immersion in 10% buffered formalin for histological studies and apoptosis detection. We followed the guidelines of the Official Mexican Standard Care and Use of Laboratory Animals (NOM-062-ZOO-1999) and the Mexican Official Standard NOM-087-ECOL-SSA1-2002-Infectious Bio Hazardous Waste - Classification and Specifications Handling. This protocol was approved by the local ethics committee (FQ/CICUAL/043/12).

3.4.1 Determination of renal function. BUN and plasma creatinine were determined using commercial kits. The activity of NAG was determined in renal homogenates with citrate buffer 0.1 M, pH 4.4 with *p*-nitrophenyl-*N*-acetyl- β -*D*-glucosaminide as a substrate (30 mM) using a *p*-nitrophenyl (PNP) standard curve. The reaction mixture was incubated for 15 min at 37 $^\circ\text{C}$ and the reaction was stopped with sodium carbonate buffer, pH 10.4. The optical density at 405 nm was measured. The data were expressed as U mg^{-1} of protein where one unit of NAG was defined as the amount of enzyme that releases 1 μmol of PNP under the assay conditions.³⁹ Plasma GPT activity was assessed by the disappearance of NADPH at 340 nm in a coupled reaction containing 1 mM GSH, 0.2 mM NADPH and 1 U mL^{-1} of GR. The protein concentration was determined by the Lowry method.

3.4.2 Histopathological analysis. The kidneys were dehydrated in ascending concentrations of ethanol, embedded in paraffin and sectioned to a 4 μm thickness, subsequently stained with hematoxylin and eosin (H&E). Slides were observed using a light microscope (40 \times) and the histological alterations

of at least 30 randomly chosen fields/slice by automated morphometry using a Leica Qwin Image Analyzer (Cambridge, England). With this equipment the percentage of damaged convoluted proximal tubules was obtained considering the histopathological changes such as swelling, vacuolization, desquamation, and cell death. In addition, the total area of the tubular epithelium was measured in microns, then the area of damaged and dead cells were determined and its percentage with respect to total area was calculated.

The histological profile of 20 proximal tubules randomly selected per rat (three rats per experimental group) was recorded, using a Leica Qwin Image Analyzer (Cambridge, UK).

3.4.3 Apoptosis detection. DNA fragmentation as an apoptosis marker was evaluated by TUNEL in renal tissue samples. 3 μm sections were deparaffinized and antigens were recuperated by boiling for 20 min in 0.01% sodium citrate solution, pH 6.0. Endogenous peroxidase activity was blocked with 3% H_2O_2 solution in methanol for 30 minutes. Antibody non-specific binding was inhibited by incubation in a 2% solution of normal swine serum in phosphate buffer saline (PBS) for 60 min. The enzyme terminal transferase was subsequently added in a 1 : 100 mixture in buffer solution (including fluorescein-conjugated oligonucleotides) to the tissues and incubated for 50 min at 37 °C in the dark. Tissues were washed 5 times for 5 min in PBS 1 \times and subsequently incubated for 30 min with the anti-fluorescein antibody at 37 °C. After washing, color was developed by adding 3,3'-diaminobenzidine and monitored using a light microscope. Slides were scanned with Aperio CS (San Diego, CA, USA) digital pathology equipment and TUNEL-positive cells were digitally analyzed in the renal cortex with an Aperio ScanScope System (San Diego, CA, USA).

3.4.4 Oxidative stress markers. MDA, 4-HNE, oxidized proteins and GSH content were measured in kidney homogenates. The MDA content was determined using tetramethoxypropane as a standard curve or renal homogenized in a solution of acetonitrile : methanol (3 : 1) with 10 mM 1-methyl-2-phenylindole in the presence of concentrated HCl and incubated for 45 min at 40 °C. After that, samples were centrifuged at 3000g for 5 min, the optical density of the supernatant was measured at 586 nm. Values were reported as nmol of MDA per mg of protein.²³ The content of 4-HNE was evaluated using the same technique used to measure MDA, but employing methanesulfonic acid with Fe(II) instead of HCl.¹⁸ The renal GSH content was obtained by forming fluorescent adducts with 1 mM monochlorobimane (33 μL) in the presence of 1 U L⁻¹ glutathione-S-transferase (GST) (33 μL).¹⁸ A standard curve of GSH (33 μL) in Krebs-Henseleit solution pH 7.4 was employed. Adducts were measured using a Synergy HT multimode microplate reader at 385 and 478 nm excitation and emission, respectively. Values are reported as nmol mg⁻¹ protein.

3.4.5 Antioxidant enzymes. The activity of GPx on renal homogenates was assessed using the technique previously described to measure plasma GPx activity with some modifications. Briefly, 400 μL of the reaction mixture was mixed with 50 μL of kidney homogenate or 50 mM phosphate buffer, pH 7.4 and 15 μL of 2.5 mM H_2O_2 . To determine the activity of GST,

20 μL of kidney homogenate was employed with a solution containing 910 μL of 50 mM phosphate buffer pH 6.5, 20 μL of 2 mM GSH and 50 μL of 1 mM CDNB. GR activity was measured based on the disappearance of NADPH at 340 nm. 50 μL of renal homogenate were mixed with 950 μL of the reaction mixture (1.25 mM GSSG, 0.1 mM NADPH, 0.5 mM Na_2EDTA in 100 mM phosphate buffer, pH 7.6). CAT activity was determined by the disappearance of 30 mM H_2O_2 (725 μL) in the presence of 25 μL kidney homogenate at 240 nm. Data were expressed as k per mg protein.⁴⁸

3.5 Statistical analysis

All the values are expressed as mean \pm standard error of the mean (SEM). Results of scavenging ability were expressed as the ability of the sample to scavenge 50% of each ROS (IC_{50}). The lower the IC_{50} values the higher the scavenging ability of the compound. Values were determined by interpolation using the least squares method calculated from three independent experiments. One-way ANOVA and Bonferroni analyses were used to compare the *in vivo* data and the Student *t*-test was used to compare the results of the histological analysis using the statistical program SigmaPlot v 11.0 (Systat Software, San Jose, CA, USA). $P < 0.05$ was considered significant.

4. Conclusion

This is the first report of the prevention of oxidative stress and decrease in the activity of antioxidant enzymes in CP-induced nephrotoxicity by a single dose of the nutraceutical C-PC. The protective effect of C-PC against CP-induced nephrotoxicity was associated with the ROS scavenging ability of C-PC. Our data suggest that the human consumption of C-PC may be useful for the prevention and/or treatment of kidney diseases associated with oxidative stress. Further pre-clinical investigation should be performed.

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